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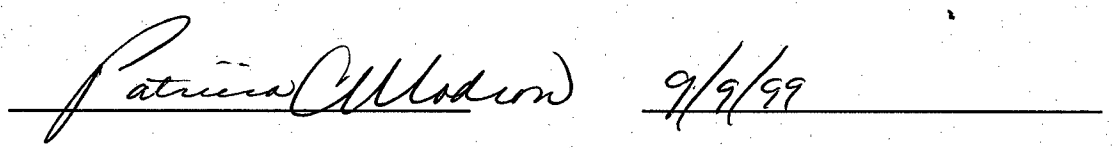

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13. ABSTRACT *(Maximum 200*

The Lewis Y antigen is a breast cancer associated carbohydrate antigen. The basis of the program was to establish and utilize structural information for Lewis Y-antibody interactions to develop novel immunotherapeutics for breast cancer treatment. It is postulated that the Lewis Y determinant on human breast adenocarcinoma cells is of key importance since it mediates internalization and lethal function of Lewis Y specific MAb. During the course of the funding period we have 1.) established the molecular recognition properties of several anti-Lewis antibodies for Lewis Y, 2.) established that peptide mimotopes of adenocarcinoma associated carbohydrate forms that include Lewis Y may function as a breast cancer vaccine, 3.) established the molecular basis for such mimicry, and 4.) have constructed a humanized single chain Fv form of a potent anti-Lewis Y antibody which is being further developed as a bispecific antibody.

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Thomas Eck Eumann

PI - Signature Date

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Introduction

The blood group-related neolactoseries carbohydrate structures Lewis X (LeX), sialyl-LeX (sLeX), Lewis a (Lea), sialyl-Lea (sLea), Lewis b (Leb) and Lewis Y (LeY) are examples of terminal carbohydrate structures related to tumor prognosis, and as such, are implicated as potential target antigens in a number of cancers (1, 2). The expression of $\alpha 1 \rightarrow 2$ fucosylated structures such as LeY, H-2 and Leb (Table 1) is inversely correlated with the survival of patients with primary lung adenocarcinoma, suggesting that these determinants promote invasiveness (1). In particular, the expression of the LeY blood group antigen in epithelial cancer tissues and cell lines has been studied using LeY-specific monoclonal antibodies (1, 3-7). LeY epitopes are expressed on MUC-1 mucins, lower m.w. glycoproteins and glycolipids, as well as higher m.w. proteins like CEA and LAMP-1 (3, 4, 8, 9). The presence of LeY epitopes on a number of different molecular carriers explains the high incidence of LeY associated with breast cancer. LeY is observed only at the secretory borders on normal tissues (7). This location appears to be inaccessible to the immune system, inducing neither tolerance nor autoimmune responses. Consequently, some Le antigens, notably LeY, are excellent targets for passive immunotherapy or a vaccine approach in the treatment of cancer.

The core components of Le antigens are structurally very similar (Table 1). These antigens constitute carbohydrate moieties of tumor associated gangliosides, the human carcinoembryonic antigen family, the human pancreatic MUC-1 antigen and are identified in carcinomas of the skin, stomach, pancreas, lung, colon, breast and prostate. The histo-blood group related antigens sLeX and sLea, are also implicated as immunogenic antigens in human melanoma (10). Melanoma patients immunized with a melanoma cell vaccine (MCV) expressing these antigens developed high titers of IgM but not IgG to both ligands. IgM titers in normal subjects were found to be low. It is noteworthy that patients who developed high titers of anti-sLe antigen IgM showed no evidence of hematologic toxicity (hemolysis, anuria or granulocytopenia) (10), despite the notion that these antigen types are displayed ubiquitously.

Table 1 Neolactoseries core antigen structures

Antigen	Structure
LeY	(Fucα1-2)Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Leb	(Fucα1-2)Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Type 1 (H-1) chains	(Fucα1-2)Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Type 2 (H-2) chains	(Fucα1-2)Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer
LeX	Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
sLeX	NeuNAcα2-3Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Lea	Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
sLea	NeuNAcα2-3Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer

Three important criteria suggest that lactoseries structures are potential targets for immunotherapy in humans: (1) their specific up-regulation (density of expression) on tumor cells; (2) their function as differentiation antigens; and (3) their role in cell adhesion and motility underlying their metastatic potential. The expression of LeX, LeY and sLeX in neutrophils is limited to humans (11). Therefore, immune responses against such carbohydrates are expected to be weaker in humans compared to other mammals. Tumor associated carbohydrate antigens (including LeY) are expressed at low levels on normal tissues. While LeY structures have not been chemically isolated from neutrophils, it is possible neutrophils do express low levels of LeY, while the expression of extended LeY with internally fucosylated structure (LeY-LeX) is limited in normal cells and tissues.

Several MAbs generated against LeY have been described in the literature, although they differ in the recognition of specific epitopes. Two antibodies called BR55-2 and 15-6A have been previously described by Dr. Steplewski (co-investigator on this project) and colleagues, that bind to the LeY antigen on breast carcinoma cells (3, 12, 13). The monoclonal BR55-2 (IgG3) directed against the LeY oligosaccharide is found to mediate ADCC (antibody-dependent cell mediated cytotoxicity) with human and murine effector cells, CDC

(complement-dependent cytotoxicity) and efficiently inhibits tumor growth in xenografted nude mice (12-14). The MAb BR55-2 was one of the first anti-LeY antibodies shown to mediate ADCC with human and murine effector cells. A limited pilot study in breast cancer patients using BR55-2 (IgG3) indicates the therapeutic potential of BR55-2 in minimal residual disease (15).

The major objective during the funded period was to elucidate structural information for Lewis Y-antibody interactions, particularly for BR55-2, in an effort to develop novel immunotherapeutics for breast cancer treatment. It is postulated that the LeY determinant on human breast adenocarcinoma cells is of key importance since it mediates internalization and lethal function of LeY specific Mab. During the funding period, our goals were to: **1.) To analyze the molecular structure of Anti-LeY MAbs and analyze their intermolecular interactions with the LeY antigen.** PCR cloning and sequencing of anti-LeY antibodies was performed to deduce the amino acid sequences of their variable regions. Molecular models of the combining region of these antibodies were generated and analyzed in a comparative fashion to identify critical contact residues of these antibodies for LeY antigen binding. This analysis elucidated binding strategies shared by anti-LeY antibodies to be correlated for the further development of anti-LeY reagents; **2.) To develop and analyze the molecular structure of LeY mimics and compare binding strategies of these mimics with LeY binding.** In our original application we anticipated developing anti-idiotypic antibodies to BR55-2 and examine their structural basis as LeY mimics. We had since changed this view to examine peptides derived from random peptide phage libraries and LeY mimics. The presentation of peptides in loop regions within the hypervariable regions of Anti-idiotypic antibodies are emulated by peptides displayed on Phage proteins. The notion of using peptide mimics of carbohydrates to induce anti-carbohydrate immune responses parallels the use of anti-idiotypic antibodies as immunogens. Utilizing Phage display, we screened for peptide clones that reacted with anti-LeY antibodies. The amino acid sequences of reactive clones that mimic Lewis Y were deduced. Structural analysis of these peptides allowed for the development of molecular models of these peptides to be analyzed in a comparative fashion with their ability to mimic Y antigen in binding assays and for complementarity to anti-LeY antibodies. This analysis provided a molecular perspective of binding strategies shared by the peptide mimotopes and the LeY antigen; and **3.) Characterize humoral responses to mimotopes of the LeY antigen.** Active immunization with mimotopes can lead to an immune response against LeY. Consequently, immunization with peptide surrogates might provide a protective immunity against breast cancer. We have examined the extent to which peptides that mimic carbohydrate subunits can induce humoral responses in mice that can target tumor cells in vitro and in vivo. We use simple peptides that mimic carbohydrate structures as models that will facilitate application of principles to eventual clinical studies in humans. This information establishes the feasibility of peptide mimotopes and design requirements for their use as potential vaccine surrogates for carbohydrate antigens in future vaccine design applications for breast cancer immunotherapy.

Body

We have published 11 papers in the last 3 years resulting from the funded studies (16-26) with 3 more in press (27-29), 2 submitted and several more in preparation. It is impossible to show all the data. The following summarizes the most salient results obtained during the current funding period.

Task 1. To analyze the molecular structure of Anti-LeY MAbs and analyze their intermolecular interactions with the LeY antigen.

1.1 Molecular recognition of LeY

Defining the configuration of native Lewis structures recognized by antibodies is important for understanding the basis for antigen specificity. We defined how BR55-2 binds to LeY (17). Our modeling study showed that BR55-2 shares similar recognition features for the difucosylated type 2 lactoseries LeY structure observed in the crystal structure of another anti-LeY antibody BR96, co-crystallized with a nonoate methyl ester LeY tetrasaccharide (30). We observed that a major source of specificity for the LeY structure by anti-LeY antibodies emanates from interaction with the β-D-N-acetyl-glucosamine (GlcNac) residue and the nature of the structures extended at the reducing site of the fucosylated lactosamine. We have shown that the nature of LeY binding is extend to the anti-LeY antibody B3 (23) (manuscript #1 in appendix). Molecular modeling of B3 complexed with the putative tetrasaccharide core of LeY was performed based upon the BR96-sugar recognition scheme as in our BR55-2 study. The B3 model emphasizes key polar and nonpolar

interactions contributing to the molecular recognition feature for LeY shared among related anti-LeY antibodies, and consistent with epitope mapping profiles of lactoseries derivatives reactive with B3 (9). The relationships among B3, BR96 and BR55-2 further allows for mutational analysis to be performed on BR55-2. Mutational studies and the generation of single chain Fvs (scFvs) of anti-LeY forms provides an enormous amount of information correlating structure/function relationship (31-36). Many mutations have been suggested for BR96 to improve its antigen affinity. One of which is a conversion of an Asp residue at position 96 to an Ala residue. Asp is found at this position in BR55-2 as well, but B3 contains an Ala. We have found that substitution of Ala for Asp in BR55-2 increases the intermolecular interaction energy of BR55-2 for the LeY tetrasaccharide core by as much as 20Kcal/mole (17). We are now evaluating the various mutations around the LeY binding site to establish the extent to which changes will increase the affinity of BR55-2 for LeY.

1.2 Development of Human scFv library.

The construction and use of recombinant chimeric and later fully humanized (CDR-grafted) antibodies to tumor-associated antigens has reduced the immune response generated to these antibodies in clinical studies. However, their long circulating half-life is a disadvantage for tumor imaging and therapy. Fragments such as F(ab')2, Fab', Fv and single chain Fv (scFv) offer increasing penetrance of solid tumors and decreasing circulating half-life, as well as reducing immunogenicity, but also lower overall tumor doses. Generally, the increased pharmacokinetic properties of smaller antibody fragments over whole antibodies have led to interest in their use for in vivo imaging of neoplastic and other diseases. In addition, their production as fusion proteins with toxins and other molecules has allowed the development of antibody-like molecules with novel effector functions. Multivalent and multispecific antibodies with defined stoichiometry could provide valuable tools for biological and medical research and for the diagnosis and therapy of cancer. Several approaches are being considered to effectively influence clearance and tumor dosage. In one approach, the tumor targeting of several novel fragments produced by chemical cross-linking of Fab' or scFv to dimeric and trimeric species has been examined.

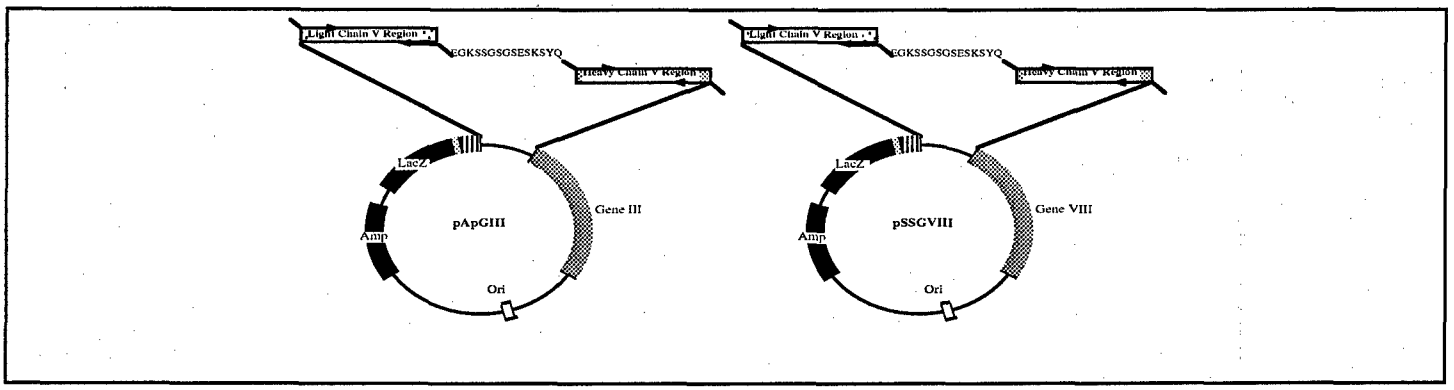
The smallest domain of an antibody necessary for antigen binding is thought to be the Fv domain. Recent advances have demonstrated that single chain antigen binding proteins (scFv) consisting of the variable domains of the heavy chain (VH) and light chain (VL) joined by short peptide linkers retain the original antigen binding properties of the Fv fragment. While LeY is considered non-immunogenic in humans in that there appears to be no evidence of circulating anti-LeY antibodies in humans, we decided to develop human antibodies reactive with LeY using a different strategy than isolating EBVtransformed human monoclonal antibodies from patients. In addition, within the scope of the grant, we wanted to make humanized single chain Fv fragments based upon BR55-2. To speed up the design process we decided we use a single chain library developed from an autoimmune patient. It was reasoned that autoimmune patients certainly generate antibodies to carbohydrates. Subsequently, it was reasoned that we might fish out reactive single chain human antibodies reactive with LeY that we can further manipulate.

In earlier studies we utilized a bacterial vector for cloning and expressing isolated human antibody heavy chain variable regions (37). Lymphocytes from patients with clinically active SLE with anti-DNA and/or anti-phospholipid antibodies were utilized to develop a single domain recombinant antibody library, and DNA binding heavy chain variable region fragments were isolated. RNA was extracted from the peripheral blood mononuclear cells (PBMC), cDNA synthesized, and heavy chain V regions amplified with human VH specific oligonucleotide primers. The VH fragments were cloned into a bacterial expression plasmid including the PelB leader peptide to direct appropriate expression. Recombinant antibodies were screened for binding to 32P-labeled double-stranded plasmid DNA, and later also characterized for binding to single stranded DNA. Binding was confirmed by standard ELISA methodology. Following our line of reasoning, we made a single chain recombinant phage library (scFv), in which PBMCs were obtained from an SLE patient with active disease (manifested by low complement levels and clinical disease activity). RNA was extracted and complementary DNA (cDNA) synthesized. Immunoglobulin (Ig)-V region sequences were amplified via the polymerase chain reaction (PCR). The PCR products were combined into an Fv region by recombinant PCR with a previously described linker peptide utilized to physically link the light chain to the heavy chain V region. This was then restriction digested and cloned into Bluescript that included PelB leader and gene VIII. This system utilizes a plasmid encoding the antibody variable regions as a fusion protein with the capsid protein(s) of filamentous bacteriophage. The antibodies are expressed as fusion proteins either with the bacteriophage gene III product (which is present in a few copies per phage particle) or the gene VIII product (which is present in

multiple copies in the phage coat). Vectors based on both gene III and gene VIII have been developed and are currently in use by our group. The vectors (with inserts) and model recombinant phage are shown in figure 1 below.

These vectors are used in combination with helper phage to produce phage-display antibodies. Following several rounds of panning and rescue of the phage, free Fv regions are made utilizing an internal stop codon in the 3' V_H primer sequence. This stop codon (amber) was deliberately included in the primer so that the plasmid can be expressed either as a fusion protein in conjunction with the gene VIII protein when grown in strains of *E. coli* that contain amber suppresser tRNA molecules, or as a secreted protein by simply growing the plasmid in bacteria lacking the amber suppresser tRNA.

Figure 1. Schematic of scFV construction



We examined the human scFv library to isolate scFv fragments that bind to LeY and to an anti-idiotypic antibody to BR55-2, called E4 (Manuscript in preparation). Ab displayed phage that bind to LeY and E4 were affinity selected by panning on antigen-coated wells. In these studies, Lewis Y antigen coupled to BSA or E4 was used to coat microtiter wells for panning. After three rounds of panning against synthetic antigen, we performed a final round of panning against E4. We then selected several clones for sequencing. In Figure 2 we compare the light and heavy chain sequences of the humanized BR55-2 (38) and two selected clones from the library. We are in the process of analyzing the structural relationships between the two species. However, it is clear that the VH and VL genes are the same. This scFv in conjunction with the humanized construct provides us with the ability to develop recombinant bispecific antibodies.

1.3 Generation of Bispecific antibodies

MAbs selected for binding to human tumors recognize surface antigens that are abundant on the tumor cells, but nevertheless are found at lower levels on normal tissues. To enhance tumor specificity, we are investigating the use of heterobispecific antibodies (BAbs) directed at two different tumor antigens on the cell surface (Manuscript in preparation). We expect that the binding of such a BAb to normal tissues will be diminished in comparison to that of a monospecific bivalent MAb. For this purpose we have generated a chemically cross-linked BAb directed to the 425 epitope on Epidermal Growth Factor Receptor (EGFR) and the BR55-2 Lewis Y epitope, both of which are overexpressed in various carcinoma cells. EGFR also forms a dimer with the Her2/neu (18) which has become an important target on Breast Tumors with the development of Herceptin. As shown in Fig. 3, this heterobispecific antibody binds with high affinity to target cells that co-express both epitopes. The binding affinity of the 425/BR55-2 BAb is somewhat greater than the affinities of F(ab')2 of either parental antibody and about 10-100 fold higher that the affinity of the BR55-2/CD3 or 425/CD3 construct that binds monovalently to target cells. These results indicate that cross-linking of distinct epitopes on the same cells by of BAbs can be accomplished that increase the efficacy of binding of BR55-2. This experiment suggests that homo and heterodimeric species of antibodies that target a variety of breast associated antigens might prove to be better immnotherapeutics. We are still pursuing the development of constructs of dimeric and trimeric single chain species of BR55-2 followed by the construction of bispecific antibodies that will include the anti-EGFR antibody 425 and an anti-neu/her2 antibody. The scFv's are being

LIGHT CHAIN

```
Hu-BR55-2  DIVNTQSPLSLPVTPGEPASISCRSSQSIVHSNGNTYLEWYLQKPGQSPQLLISKVSNRFS
HU-amhk13  DIVMTQSPSSLSASVGDRVTITCRAS    H NIIDYLSWYQQKPGKGPNLLIYAASRLQS
E419A      DIVMTQSPSSLSASVGDRVTITCRSSQTI R N   YLNWYQQKPGKAPKLLIYDASSLQS

HU-BR55-2        GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGSHVPFTFGQGTKLEIK
HU-amhk13        GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSHFSPYTFGQGTKLEIK
E419A            GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSSPLTFGPGTKVDIK
```

HEAVY CHAIN

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HU-BR55-2        EVQLLESGGGLVQPGGSLRLSCAASGFTFSDYYMYWVRQAPEKRLEWVA
HU-amhk13        EVALVDLGGNVVQPGGSLRLSCAASGFTFHDYTMHWVRQAPGKGLEWVS
E419A            EVALLVEWGAVVRPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS

HU-BR55-2        YISNGGGSSHYVDSVKGRFTISRDNSKNTLYLQMNSLRAEDTALYH
HU-amhk13        LISWHGHSTFYADSVKGRFTVSRDNSKYSLYLEMNSLTTDDTALYY
E419A            TISFSGSSTYHADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY

HU-BR55-2        CARGMDYGA          WFAYWGQGTLVTVSS
HU-amhk13        CAKEVSPSDTAMVTFRYHDAFDSWGQGTMVIVSSASTK
E419A            CAKAPLGDY      LWGSYPLDYWGQGTLVTVSSASTK
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Figure 2 Sequence of the light and heavy chain of isolated human scFv reactive with LeY.

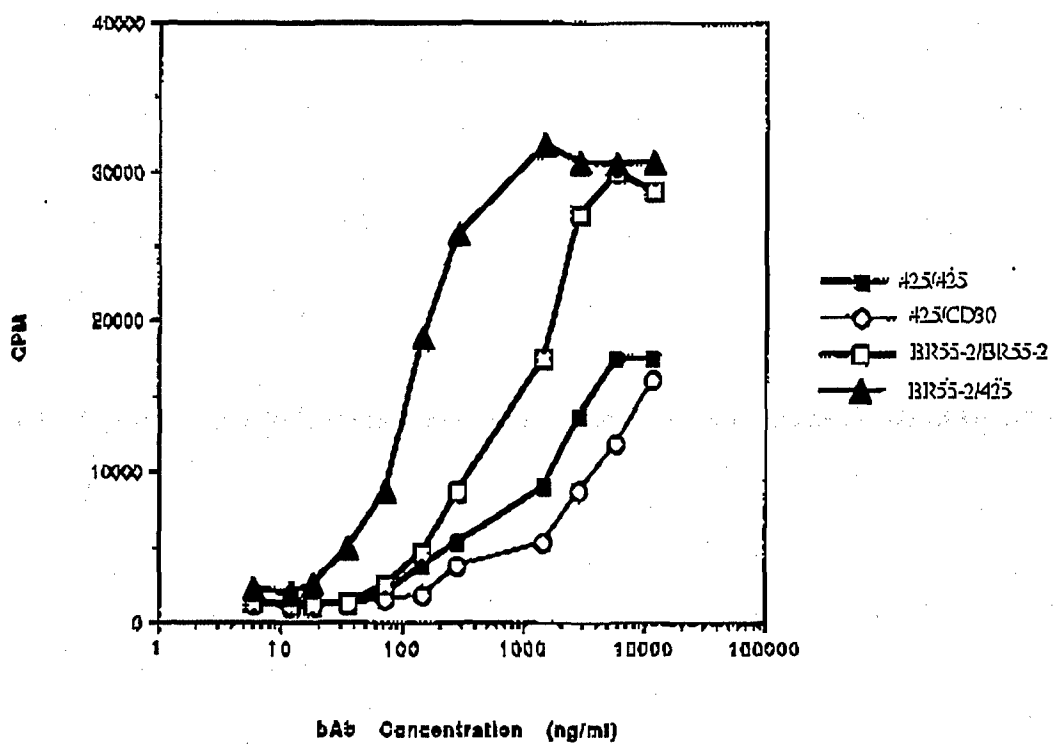


Figure 3: Binding of chemically crosslinked BAbs to MCF-7 breast cells as determined in indirect radioimmunoassay. Enhanced target cell recognition of heterobispecific BR55-2/425 is shown in comparison to 425/425 and BR55-2/BR55-2 F(ab')2 and BR55-2/anti-CD3 F(ab')2.

constructed and expressed in bacteria to facilitate genetic manipulation necessary to produce the variety of antibody-form derivatives discussed in this proposal and to allow for rapid modifications of determinants which may affect protein folding, binding, and/or bioactivity. Additional advantages of bacterially produced antibody forms over standard hybridoma techniques include: increased stability and consistency of protein production, high protein yields, and increase ease of culturing. Ultimately this approach has the greatest flexibility and potential. We are planning to construct radioconjugates of these molecular species and explore their biodistribution in mice (29).

Task 2.) Analyze the molecular structure of LeY mimics and compare binding strategies of these mimics with LeY binding

2.1 Molecular mimicry of Lewis Y

In our studies we observed that the model of BR55-2 and B3 emphasizes the role played by the fucose methyl groups facing the floor of the antibody combining site. These methyl groups might be mimicked by methyl group containing amino acid residues in a designed peptide or anti-idiotypes that compete with LeY for anti-LeY antibodies. A peptide that mimics the LeY antigen has been described (39) that contains such methyl groups. The anti-LeY antibody B3 was found to bind to the peptide sequence APWLYGPA presented on phage display in which the putative sequence APWLY is critical for binding to the antibody (39). The B3 antibody displays homology with other anti-LeY antibodies, including the recently crystallized antibody BR96 co-crystallized with a nonoate methyl ester LeY tetrasaccharide. The tumor cell binding specificity's of B3, and BR55-2 are different. As with BR55-2, molecular modeling of B3 complexed with the putative tetrasaccharide core of LeY was performed based upon the BR96 -sugar recognition scheme (17, 23). While current procedures for predicting ligand - antibody interactions is limited, mainly due to the conformational flexibility of ligands and antibodies, and the role of solvent in mediating ligand recognition and binding, the utilization of a crystallographically determined starting position can lend to discriminating differences in binding orientations of analogues. Utilizing the positional information of the LeY structure, we implemented the program Ligand-Design (LUDI (40) Biosym Technologies) to search a fragment library to guide in the position of the putative APWLY peptide sequence (manuscript #1 appendix). Optimization of the positioned peptide indicates structural similarity between the carbohydrate tetrasaccharide core of LeY, with similar functional groups on the B3 structure in contact with the peptide. This analysis therefore provides a unique perspective of how a peptide sequence fits into the antibody combining site, competing with a native antigen.

In the placement of the B3 reactive putative peptide sequence APWLY, we made use of the program LUDI to identify compounds that potentially interact with the B3 combining. Over 260 fragments were identified for the model, with the largest radius of interaction, with most redundant for the same set of potential hydrogen bond donors or acceptors on B3. In evaluating the fragments we compared fragments identified by LUDI relative to the APWLY sequence such that the fragments could occupy non-redundant sites and be spatially far enough from each other to accommodate the peptide backbone. LUDI found that a Trp like residue forms a hydrogen bond with the backbone carbonyl oxygen of Trp H98, that a lipophilic residue representative of a Leu side chain is bounded by residues Val L94, His L27D, and Ala H58 another lipophilic residue representative of an Ala and Pro side chain is bounded by Ala H97.

The APWLY sequence was then modeled such that the corresponding Trp, Pro, Leu and Ala residues occupied relative positions as the identified LUDI fragments. In affect one wants to "stitch" the fragments together to form a peptide. We modeled the peptides two ways. The first, was to use individual amino acid fragments oriented with their side chains superimposed on the LUDI identified side chain types. The individual fragments were then restrained to form concomitant backbone geometry's and conformations. As expected, such an approach resulted in highly strained conformations. Alternatively, a peptide was built and the phi, psi angles rotated until the respective side chains were in close proximity. The positioned peptide fragment-B3 complex was then energy optimized with a restrained dynamics calculation. After this dynamics run, the complex was again energy optimized to convergence without the imposition of constraints. Deviation of the backbone conformation of the peptide-B3 complex relative to the respective LeY-B3 complex was found to be only 0.29 A. This indicates that the placement of the peptide within the antibody combining site did not dramatically alter the overall conformation of either B3 structures.

While the LUDI search provided a favorable geometry for peptide side chain placement, the final placement of the peptide side chains within the antibody combining site relative to the LUDI positioned fragments were different. Several different starting geometry's for the peptide placement in the BR96 model were tested. Intermolecular interaction calculations indicate that the majority of the peptide binding comes from dispersion interactions. Five potential hydrogen bonds were found for the most stable of the models. One involves the N7 of Trp interacting with the backbone carbonyl group of Trp H98, the carbonyl backbone of Trp interacting with His L27D, the Tyr side chain hydroxyl group interacting with hydroxyl group of Ser H55, the backbone carbonyl group of Ala interacting with Asn H52A, and Tyr H33 side chain interacting with the carbonyl backbone of Leu, whose hydrophobic side chain being further stabilized by dispersion interactions with Val L94. We have further constrained the model peptide to form a beta turn in which a hydrogen bond is potentially formed between Tyr amide and Pro carbonyl groups.

In this positioning we observed that the Ala-Pro residues of the peptide occupied a similar position as the LeY GlcNAc residue. This positioning indicates that the proline residue mimics the spatial position of the glucose unit of GlcNAc, while the Ala methyl group is positioned similarly as the terminal methyl group of GlcNAc's N- acetyl. The Trp residue occupies a volume associated with the cFuc residue, and the Leu residue occupying the volume and the hydrophobic interaction of bGal. The Tyr residue occupies a position not associated with the LeY binding to B3.

The search of a fragment library for possible compounds that would fit within the B3 combining site, provides a guide to position the side chains of the putative peptide sequence APWLY to effectively compete with the LeY antigen . The binding mode of the peptide did not faithfully mimic the LeY antigen in contacting all the same functional groups on B3, but binds in a fashion that provides for at least steric competition between the peptide and the LeY structure. We observe that the peptide might be availed to form a beta turn in the binding site. This conformation lends itself to the Tyr residue of the peptide to potentially interact with several residues in CDR2 of the heavy chain of B3 which include Asp H53, Ser H52, Ser H55 or Ser H56. These residues are different with respect to BR55-2 which does not effectively bind the APWLYGPA peptide. It is noted that the positioning of the peptide within the B3 combining site is strictly a model and awaits information from crystallographic studies on related anti-LeY reactive antibody-peptide complexes. Nevertheless, the model does suggest that peptides that contain the APWLY motif should bind to B3, effectively mimicking the LeY antigen as observed experimentally. The extent of potential fragments that we have found to interact with the B3 combining site indicates that there may still be many ways for peptides with differing sequences to interact with B3 in spite that only one peptide sequence was identified in the phage screening (39).

2.2 Identification of BR55-2 binding site residues

The molecular nature of mimicry between carbohydrates and peptides is still not well understood. Studies on carbohydrate mimicking peptides and their cognate antibodies suggest that aromatic-aromatic and hydrophobic interactions are critical chemical forces which modulate binding(16, 39, 41-46). Peptide mimotopes for carbohydrates have been defined containing a two aromatic amino acid repeat motif W/YXY found to bind to Con A (YPY) (41, 42), in peptides that mimic the Lewis Y antigen (WLY) (39), in peptides that bind to anti-Crytococcous polysaccharide antibodies (45) and that mimic the major C polysaccharide of *N. Meningitis* (16). As previously noted (45), these observations suggest that a particular peptide structure is required for polysaccharide mimicry (47). The role played by the aromatic rings might be to position particular residues for reactivity or they may directly bind to the antibody (39). We and others showed that peptide mimotopes either containing the W/YXY sequence tract or are homologues of the motif can adopt β turns in the antibody combining site (16, 23, 48, 49).

To better understand these interactions, we have been studying the structural basis of LeY binding to antibodies (17, 23). Defining the configuration of native carbohydrate structures recognized by antibodies is important to adequately assess the extent to which the same mechanisms for binding are used by peptide mimotopes (48). Structural studies on Lewis antigens have generally substantiated that their conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes (17, 50-52). These results further indicate that hard sphere or rigid-geometry calculations, albeit without solvent, provides a good picture of the steric repulsion that modulate the conformational properties of the Lewis antigens. Consequently, structure-based drug design approaches (40, 53, 54) offers the ability to establish potential

interaction profiles (epitope map) to elucidate a molecular basis for peptide mimicry of the LeY antigen in binding to BR55-2 and related anti-LeY antibodies.

We wished to determine whether this model could be used to ascertain the extent to which peptide mimotopes of LeY can be structrally correlated with LeY binding for BR55-2. Our goal was to determine if cross-reactive peptides recognized by BR55-2 bind by the same mechanism as LeY; if so the basis of cross-reactivity would be structural. Towards this end we described the structural basis for the antigenic mimicry by some peptides for the LeY antigen (Manuscript #2 in appendix; submitted). A structure-based computer screening approach was used to assemble an epitope map of potential amino acids that could interact with the BR55-2 combining site. To test this idea we performed a computer screening search with the computer program LUDI that resulted in 231 ligands identified to interact with the BR55-2 combining site. Table 2 summarizes the hydrogen bonding profiles of ligands for BR55-2, and identified contact residues within the BR55-2 combining site. The majority of contacts with LeY occur with main chain (MC) atoms, with some involvement with side chains (SC), as does interaction with the LUDI identified ligands. Of note, all BR55-2 residues that interact with LeY are identified in the LUDI search. Guanidinium type groups are observed to form bifucated hydrogen bonds with the same BR55-2 residue functional groups as the Fucα(1-3) moiety of LeY.

Table 2 Ligand/Epitope Hydrogen Bond Contact Residues on BR55-2

	BR55-2	
Amino Acid Types Identified Computationally	***Light Chain***	***Heavy Chain***
Guanidinium		Ala100(MC), Met96(MC), Tyr35(SC) Asn52A(SC), Asp31(MC) Ala 100(MC), Met96(MC) Asp97(MC), Met96(MC)
Tyr	Ser92(MC),	Tyr35(SC), Asp31(SC), Asp31(MC) Asp97(MC), Ser92(MC)
Trp		Ala100(MC), Met96(MC), Tyr33(SC) Asp31(MC), Asn 52a
Ser/Thr	Ser27E(SC)	Asp31(MC), Asp97(MC), Tyr50(SC)
His		Tyr32(SC), Tyr35(SC), ASP97(MC) Asp31(MC), Tyr50(SC)
NH/CO	His27D, Tyr32(SC)	Tyr35(SC), Asp31(MC), Gly53(MC) Tyr32(SC), Asn52a(SC)
LeY tetrasaccharide Identified Crystallographically		
Gal	His27D	Tyr35(SC)
GlcNAc		Tyr33(SC)
Fucα (1-2)	Ser27E	
Fucα(1-3)		Ala100(MC), Met96(MC), Tyr35(SC)

As a further illustration of this approach, definition of residue types shown in Table 2 can be combined with bulky hydrophobic amino acids occupying the LeY spatial volume. In figure 4a, representative non-overlapping organic ligands are shown positioned within the BR55-2 combining site relative to LeY. It is observed that Leu like groups can occupy the spatial volume of Fucα(1-3) and Gal moieties of LeY. A hydroxyl type residue is hydrogen bonded Asp 31 (MC) of the heavy chain, with a Trp like ligand hydrogen

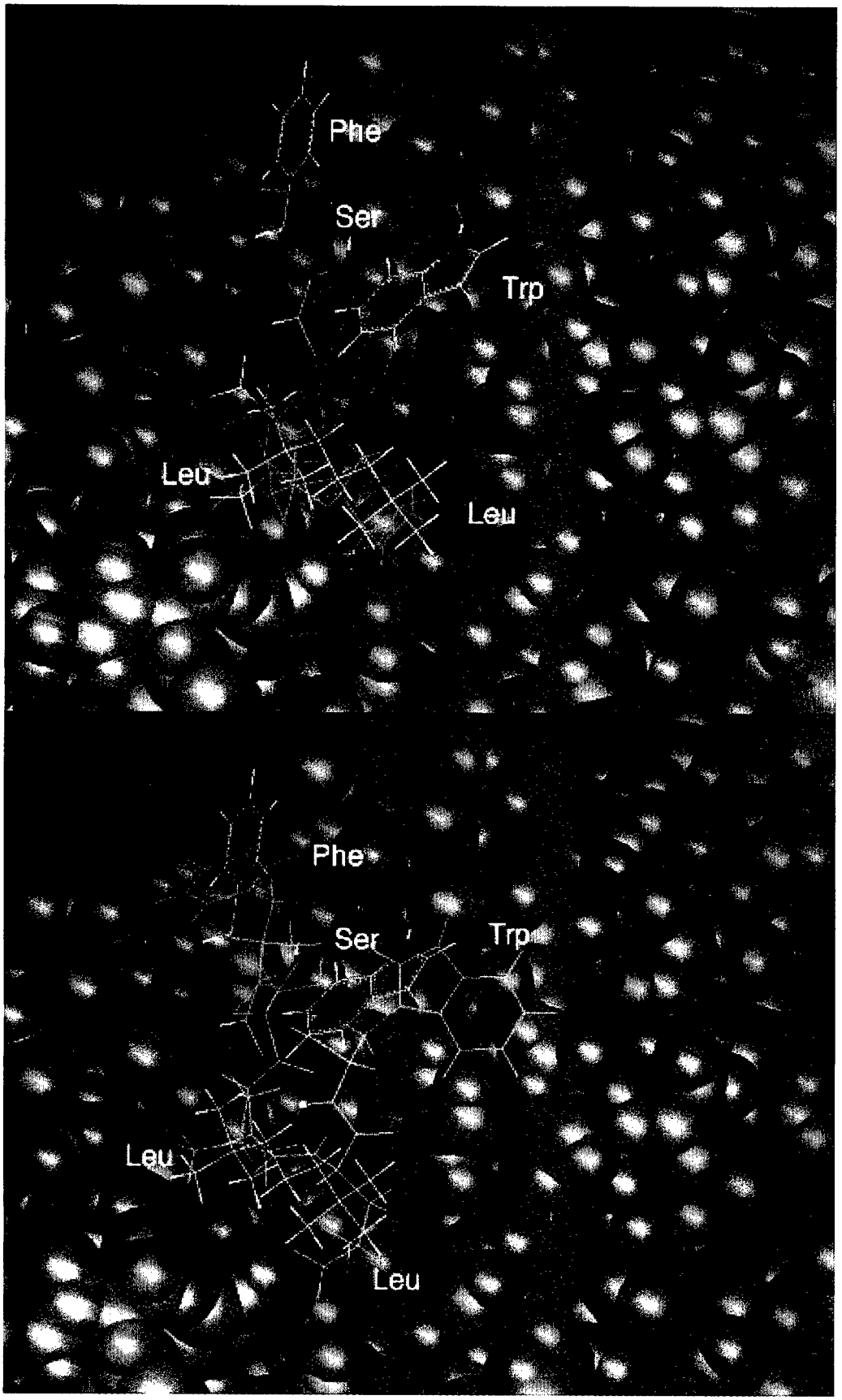


Figure 4

bonded with the side chain of Asn 52A of the heavy chain side. The Phe, Ser and Trp like ligands occupy the volume of the GalNAc moiety.

These relative positions can be used to form a putative peptide with the BR55-2 sequence FSLLW within the BR55-2 combining site as shown in Figure 4b in which the Ser and Trp hydrogen bonding schemes are retained. This binding mode conformation is similar to turn type conformations previously suggested for peptide binding to antibodies (16, 23, 48, 49) that serves to place the peptide sequence into a depression in the antibody combining site. Previous studies implicated the aromatic homologous putative sequence tracts WLY, WRY and YRY as mimics of the LeY antigen (25, 39). Conformational studies indicate that the W/YR/LY and W/YPYmotifs can adopt beta turn type structures (16, 23, 49) suggesting a particular peptide structure is required for polysaccharide mimicry. The beta turn characteristics of mimicking peptides has been further implicated as a binding mode conformation as evidenced in a crystal structure of a peptide mimic of a cryptococcus carbohydrate epitope in complex with an anti-cryptococal antibody (48). Root mean square deviation (RMS) of the BR55-2-beta turn peptide complex after minimization and dynamics calculations was found to be only .52 A compared with the BR55-2-LeY complex, indicating that the beta type II turn is readily accommodated within the BR55-2 combining site.

2.3 Reactivity of peptide mimics with BR55-2.

The LUDI results indicate that a variety of amino acid residues can certainly be found to interact with BR55-2, containing combinations of aromatic, bulky hydrophobic and hydroxyl containing residues. To further substantiate this conclusion, we screened a phage library containing a random 15 amino acid insert with BR55 (24 and manuscript in preparation). The ability of an antibody to react specifically with a carbohydrate epitope depends on the exposed nature of the terminal subunits and the accessibility of core carbohydrate structures. The role of core carbohydrate structures is to stabilize and impart the spatial disposition of branching terminal subunits. The degree of accessibility of core structures is most likely dependent on the nature and complexity of the branched subunits. In terms of mimicry, several possibilities exist. For example, mimicry is dependent on the spatial distribution of carbohydrate functional groups. The more branched a complex carbohydrate is, the more likely the combinatorial spatial disposition of functional groups might lead to cross-reactivity. This result might also decrease the accessibility of core structures. Likewise, the smaller the carbohydrate or less branched and complex, the more likely that the core structure is exposed and the combinatorial spatial disposition of functional groups decreases.

Our modeling studies on BR55-2 and B3 indicate that they contact LeY in a similar fashion, albeit perhaps with differing affinities. In the context of peptide mimicry, of interest is whether peptides mimic the conformational properties of carbohydrates in that they bind to the same amino acid contact sites on the antibody or that they interact with alternative contact sites and mimicry is really the result of steric inhibition. We observed that a peptide identified to bind to B3 does not bind to BR55-2 by ELISA, nor inhibits BR55-2-LeY interaction. This facet suggests that peptides identified by phage display can be specific for their isolating antibody. Subsequently, it is possible that peptides identified by phage display that are considered cross-reactive with the anti-carbohydrate antibody, are not necessarily recognized by the same mechanism as their carbohydrate counterparts, even though their binding sites are overlapping or adjacent.

To further explore the nature of the antigenic and immunogenic properties of such mimotopes, synthetic peptides with aromatic amino acids were tested to delineate reactivity patterns with several anti-neolactoseries monoclonal antibodies (MAbs) (27) (Manuscript #2 and #3 and in appendix). We synthesized respective multiple antigen peptide (MAP) forms of various peptides for detection of reactivity patterns with BR55-2. Results by ELISA demonstrate that the MAbs can distinguish particular peptide motifs that include the sequences GGIYYPYDIYYPYDIYYPYD, GGIYWRYDIYWRYDIYWRYD, and GGIYYRYDIYYRYDIY YRYD (Table 3). Evidence for Arg recognition by BR55-2, as suggested in the Ludi search, is demonstrated in ELISA assays in that substitution of Pro for Arg within the YRY sequence tract diminishes BR55-2 reactivity for the YPY containing peptide (Figure 5). The Arg containing peptides K61106 and K61107 (Table 3) bind to BR55-2 and compete with LeY for BR55-2 in a concentration dependent manner (Figure 5b), while the Pro substituted peptide K61105 (Table 3) displays diminished ELISA reactivity with BR55-2 (Figure 5a) and does not compete with LeY for BR55-2 binding (Figure 5b). These results indicate that a single substitution within a peptide sequence effects the antigenic mimicry of what are otherwise homologous peptides and that the presence of aromatic groups alone does not account for cross-reactivity.

Table 3 Peptides Described in These Studies

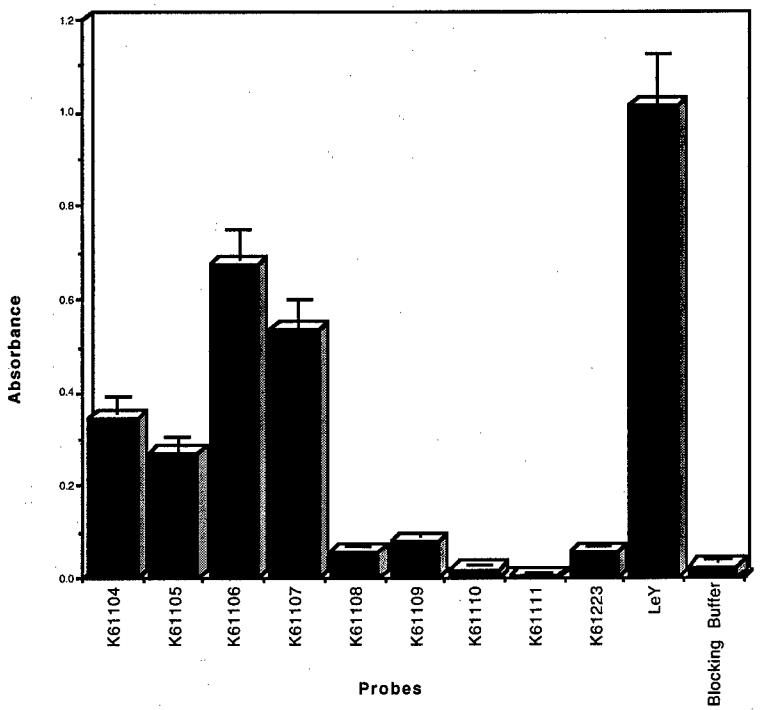
Designation	Sequence Motif	Structure	Method of Identification	Binding	Inhibition
K61105	YPY	GGIYYPYDIYYPYDIYYPYD	Synthetic Design	+	-
K61106	WRY	GGIYWRYDIYWRYDIYWRYD	Synthetic Design	++	++
K61107	YRY	GGIYYRYDIYYRYDIYYRYD	Synthetic Design	++	++
K61108	WLY	GGGAPWLYGGAPWLYAPWLY	Synthetic Design	-	-
K61223	WLY	GGGAPWLYGAPWLYGAPWLY	Synthetic Design	-	-
K61109	WRY	GGARVSFWRYSSFAPTY	Phage	-	-
K61110	WPY	GGGWPYLRFSPWVSPLG	Phage	-	-
K61111	WVF	GGAGRWVFSAPGVRSIL	Phage	-	-
K61104	FSLLW	IMILLIFSLLWFGGA	Phage	+	++

Binding is binding to BR55-2. Inhibition is inhibition of LeY binding to BR55-2

The effect on binding with changing the presentation of a putative sequence tract is further observed with the peptide K61109 (Figure 5a) which contains the WRY sequence tract (Table 3). The general lack of reactivity of aromatic residue containing peptides is also observed with peptides K61108, K61110, K61111 and K61223 (Table 3). The peptides K61110 and K61111 were isolated from a 15mer library with BR55-2. The initial choice of using the 15 mer library was predicated on the notion that this length is similar to complementarity determining regions (CDR) in antibodies which confers mimicry capacity to many anti-idiotypic antibodies. Isolation of 100 random clones resulted in the identification of a variety of peptides containing these amino acid compositions. We identified 28 peptide families with selected family sequences shown in Table 3. One peptide isolated displayed an exact match of the putative FSLLW sequence track (K61104 Table 3), while another contained a WRY sequence (K61109, Table 3). It was previously noted that it may be that library screening processes favor peptides that can assume conformations conducive to antibody binding when expressed on phage, but do not achieve the same conformation when synthesized free of the constraints imposed by the phage protein carrier (45, 55). In contrast, the peptide K61104, also isolated with BR55-2, displays reactivity with BR55-2 in ELISA and can compete as effectively as the K61107 and K61106 peptides for LeY binding to BR55-2

Because the FSLLW sequence tract was identified both computationally and by phage display, we decided to test the immunological mimicry of the K61104 peptide, Balb/c mice were immunized with the K61104 MAP peptide administered with the adjuvant QS-21. The anti-K61104 sera was predominately of IgM isotype as observed in our previous studies using MAP peptides (27). The anti-K61104 sera displayed a three to fold increase in reactivity for LeY over Leb, titering up to 1:2000 in ELISA (Figure 6a). At 1:50 serum dilution (Figure 6b), higher levels of reactivity are observed for LeY and LeY substituents with about the same level of reduced reactivity for Leb hexasaccharide, LeX-pentasaccharide, sLeX, Lea, and sLea. Minimal binding is observed for a ubiquitous disaccharide unit Galβ1-3Gal.

To further define the minimal determinate that distinguishes selectively for LeY over its homologues, the serum was further screened against a variety of LeY constituents with the best reactivity observed with the Fucα1-3GlcNAc moiety (Figure 6b), reflecting the spatial association of the peptide for BR55-2 in comparison with other moieties on LeY (Fig 4b). Most importantly, the anti-K61104 sera distinguishes the Fucα1-3 from the Fucαa1-4 GlcNAc linkage, displaying significantly reduced reactivity with Fucα1-4GlcNAc. This selective interaction sets apart reactivities between Leb and LeY, since reactivity is observed for the H type 1 constituent of Leb (Figure 6b). The cross-reactivity of the anti-peptide serum for LeY in a specific manner suggests a structural mimicry between the K61104 peptide and LeY as indictaed in Figure 4.



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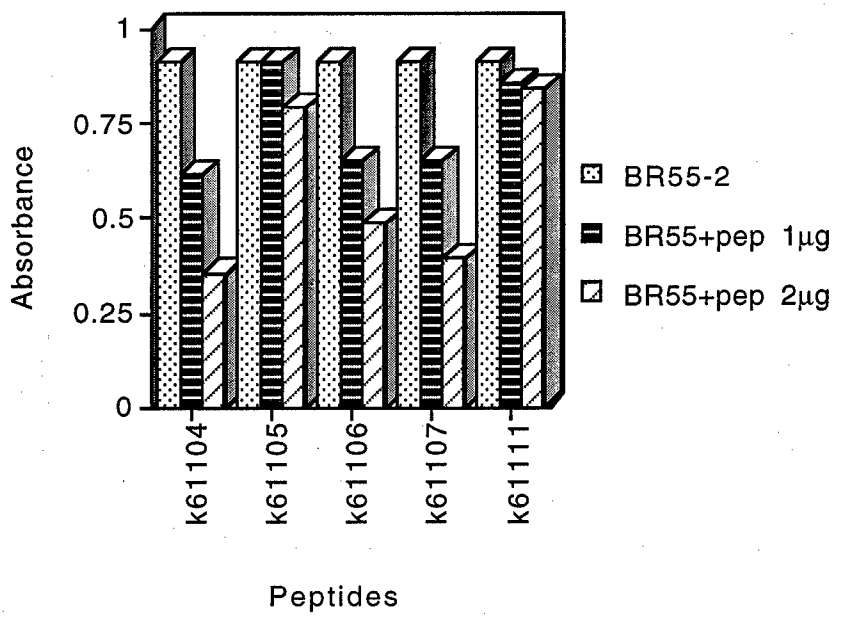


B.

Figure 5. Reactivity of putative motifs by ELISA. (A) Reaction of BR55-2 with respective MAP peptides. (B) Inhibition of MAb BR55-2 binding to solid-phase LeY-PAA by soluble MAP peptides. A constant amount of BR55-2 was incubated with increasing amounts of MAP peptides, and then reaction of free MAb with LeY was measured by ELISA. Data points reflect 50% inhibition at 2.3 uM of K61104 peptide, 1.6 uM for K61106 and K61107 pepide inhibitors as measured by reduction of Absorbance value in ELISA.

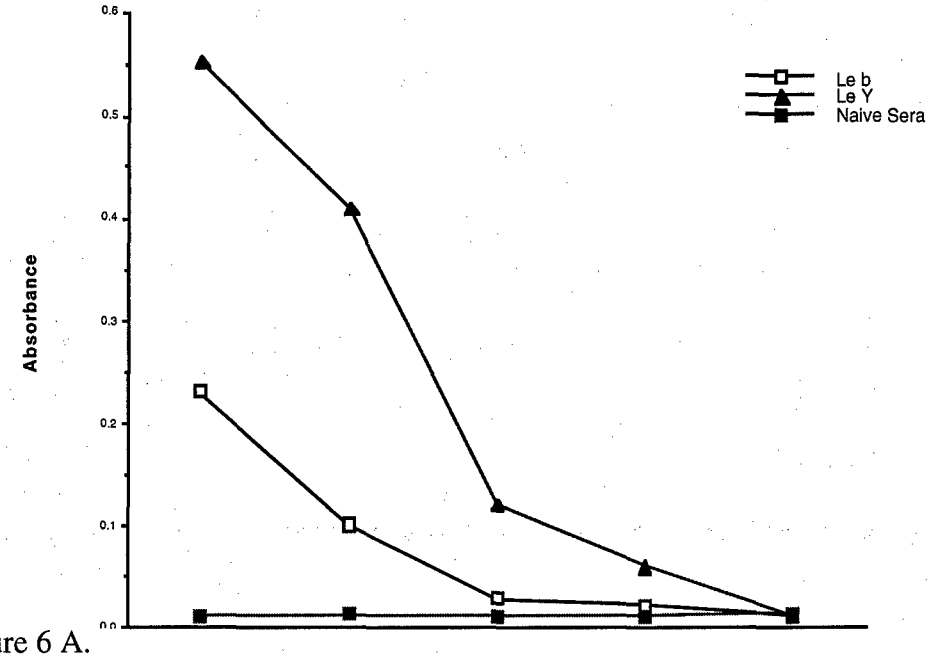


Figure 6 A.

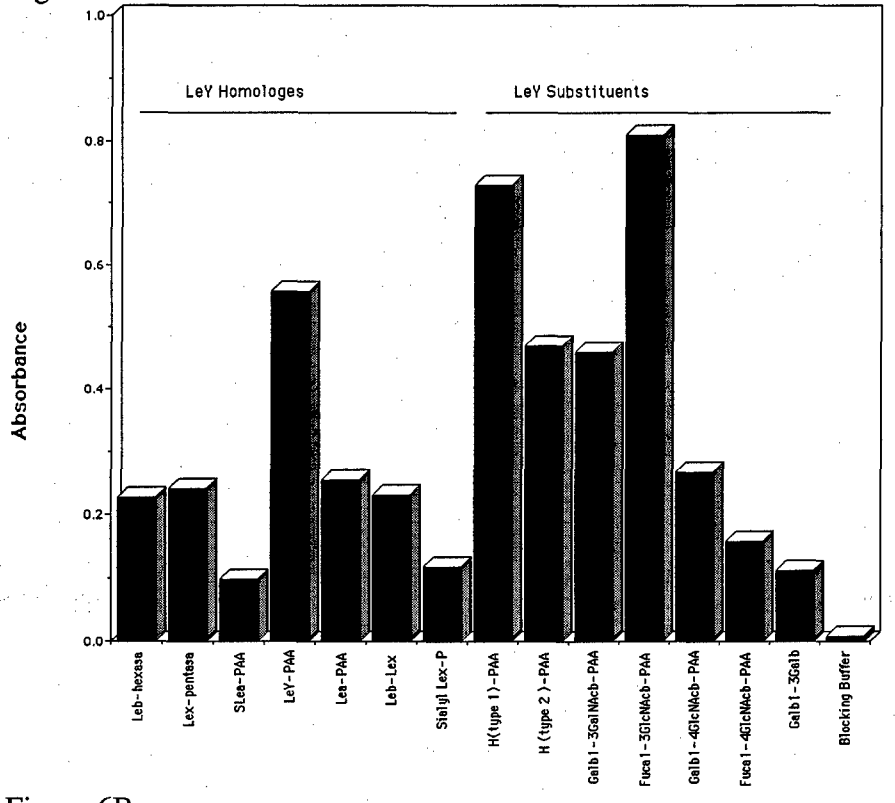


Figure 6B.

Figure 6. Reactivity of the anti-K61104 serum with neolactoseries constituents. A.) Titration of the IgM portion of the derived anti-peptide sera with LeY and Leb. B.) Profile of cross-reactivity of anti-peptide MAP sera with Lewis carbohydrate probes.

Task 3.) Characterize humoral responses to mimotopes of the LeY antigen.

3.1 In vitro and in vivo functionality of Le antigen mimicking peptides

Very few groups are investigating carbohydrate based vaccines or carbohydrate based immunotherapy. One major reason for this is that carbohydrate antigens are expensive and very difficult to synthesize. Further, expression of tumor-associated carbohydrate antigens is by no means specific to tumors. Crucial issues are expression of antigen density, multivalency, reactivity threshold of antibody binding, and efficient production of antibody having a strong complement-dependent or T cell dependent cytotoxic effect on tumor cells without damage to normal tissues. Studies on cancer vaccine development depend on many factors for success that include: 1) Selection of carbohydrate epitopes; 2) Design and assembly of epitopes coupled to macromolecular complex as an efficient immunogen; 3) establishment or availability of a good animal model; 4) Evaluation of immune response in animals; tumor rejection without damage to normal tissues; and 5) careful clinical application. Since carbohydrate antigens are generally weakly immunogenic in humans, only short lived IgM responses have been historically observed. The importance of adjuvant sublimation is highlighted in such studies to offset the relatively weak immunogenicity of the carbohydrate structures. In addition, antibodies to carbohydrates are typically of low affinity and the notion of how cellular immunity is modulated by carbohydrates is unclear.

While a LeY-conjugate vaccine is attractive for development, antibodies generated against synthetic LeY are not always cross-reactive with native LeY antigen forms (56). This general phenomenon has also been observed with sialyl-Tn (sTn) formulations, suggesting that neoglycoproteins containing sTn in which the carbohydrate structures are clustered together would make better immunogens (57, 58). This has also been observed with GM2-KLH formulations in which the majority of IgG antibodies induced by the GM2-KLH/QS-21 vaccine while reactive in ELISA, failed to react with cell surface expressed GM2. So while synthetic LeY formulations induce anti-synthetic LeY reactivities, the generated antibodies may not bind to tumor cells or bind very weakly. This observation formulates one of the rationales for our studies. The basic hypothesis of this application is that peptide mimotopes can induce functionally relevant immune responses **in in vivo and in vitro models.** (26) (Manuscript #4 in appendix) Aromatic-aromatic interactions appear to mimic a variety of carbohydrate subunit interactions (Table 4). We have shown that peptides containing such motifs induce sera that is highly functional both in vitro and in vivo.

Table 4. Peptide motifs that mimic carbohydrate structures

Motif	Carbohydrate	Structure
YYPY	Mannose	methyl-α-D-mannopyranoside
WRY	Glucose	α(1-4)glucose
PWLY	Lewis Y	Fucα1→2Galβ1→4(Fucα1→3)GlcNAc
YYRYD	GroupC Polysaccharide	α(2-9)sialic acid

We showed in vivo protective immune responses in peptide immunized mice against a lethal challenge of Neisseria meningitidis (16). The immunizing peptide (peptide-proteosome conjugates) mimics the major group C meningococcal polysaccharide (MCP) (16). We reported that peptides containing aromatic motifs (Table 4) can effectively mimic mannose, sialyl and histo-blood group related carbohydrate epitopes (particularly LeY) expressed on breast tumor lines (21) and found on the major envelope protein of the human immunodeficiency virus (HIV) (25). The basis for these studies was our observation that the LeY tetrasaccharide structure is similar to the core structure of MCP, suggesting that it is possible for antibodies to cross-react with these two moieties. In the former studies we have found that carbohydrate-mimicking peptides retain carbohydrate-like conformations, inducing anti-carbohydrate immune responses against breast tumor cells and mediating their killing by a complement-dependent mechanism. Anti-LeY antibodies have been previously shown to neutralize HIV-1 infection in vitro (59). Consequently, we asked whether sera reactive with LeY expressing cells will also react with the envelope protein of HIV-1. The **in vitro** neutralization of HIV-1 infection of target cells was

specific and similar to human sera from infected subjects. The neutralization pattern could be modified by changing one amino acid in the immunizing peptide (25).

Serum against all three peptides displayed reactivity with synthetic histo-blood group related antigen probes. Immunologic presentation of the peptides as multiple antigen peptides (MAPs) improved peptide ability to induce LeY specific immune responses. Serum bound to human tumor cells that preferentially expressed neolactoseries antigens, but not to normal tissues. Immunoprecipitation of human breast tumor cell lysates before and after treatment with tunicamycin confirmed serum carbohydrate binding peptides (27)(manuscript #3 in appendix). The anti-peptide sera mediated tumor cell killing by complement mediated cytotoxicity (manuscript #3 in appendix). These results indicate that mapping peptide epitopes with anti-carbohydrate antibodies can lend to defining antibody fine specificities that can go undetected by screening of carbohydrate antigens alone. In addition, these results confirm that peptides and carbohydrates can bind to the same antibody binding site and that peptides can structurally mimic salient features of carbohydrate epitopes.

3.2 Immunogenic mimicry of peptide motifs

The neolactoseries structures LeY, LeX, sLeX, Lea, sLea, and Leb all share a common epitope topography (50, 60, 61). Multiple antigen peptides, shown in Table 4, generate serum that is cross-reactive with the common topography of the neolactoseries structure, yet displays a higher avidity for LeY (21, 25, 27). These anti-peptide serum antibodies are primarily of IgM isotype that are specific for neolactoseries expressing cell lines and human tumors, while displaying little reactivity with non-neolactoseries expressing cell lines and human tissues (27). Further analysis of anti-peptide serum binding is observed to a variety of human LeY expressing tumor lines that include human breast carcinoma lines SKBR3 and MCF7, and the LeY-sLeX expressing human prostatic line PC-3 as assesed by FACs analysis (Table 5) (manuscript #5 appendix submitted). Serum is also cross-reactive with the murine fibrosarcoma cell line Meth-A which expresses sLeX. Minimal reactivity is observed for serum with the LeY negative human melenoma line SK-MEL-28 which is GD3 positive and LeY negative. Some cross-reactivity with this cell line has been observed previously with serum raised to LeY-conjugate forms (62). These data reconfirm that the peptide mimotopes effectively immunologically mimic the neolactoseries structures, inducing a humoral immune response cross-reactive with neolactoseries expressing human tumor cell lines.

Table 5 Binding of Various Anti-Peptide Sera and Monoclonal Antibodies to Neolactoseries Expressing Cell Lines As Measured by FACS

Cell Lines	Anti-1104	Anti-1105	Anti-1106	Anti-1107	FH6	ME361	BR55-2
SKBR3	443/138*	326/128*	418/133*	445/140*	ND	9 (0.1ug)	144 (0.1ug)
MCF7	560	593	595	538	42(0.5ug)	ND	352
PC-3	118	141	129	330	420 (0.1ug)	14 (.1ug)	28(0.1ug)
Meth-A	183	87	124	143	430 (0.1ug)	15 (0.1ug)	15 (0.1ug)
SKMEL-28	ND	47.0	33.0	49.8	ND	26 (0.1ug)	7 (0.1ug)

Final Sera Concentration is 1:50. Background Mean Fluorescence associated with non-specific mouse sera is on average 24.2. The asterisks indicates IgG portion of anti-peptide serum reactive with SKBR3 cell line. ND is not determined.

3.3 Tumor cell cytotoxicity

Antibody mediated cell killing is one mechanism for tumor cell destruction. Anti-carbohydrate antibodies might mediate complement-dependent-cytotxicity (CDC) better than cytotoxicity associated with various effector cells (63). To assess the functionality of the anti-peptide serum against tumor cells, we examined CDC mediation of serum in the presence of human complement, targeting the LeY positive MCF7 human breast adenocarcinoma cell line (Figure 7) (manuscript #5 in appendix). Serum raised to a pentasaccharide LeY-conjugate mediates the killing of this cell line in vitro out to 1:80 titer (62). Similarily, detectable lysis was observed titering up to 1:80 serum dilution for serum raised against the peptide mimotopes, and against synthetic LeY-PAA (Figure 7). The anti-K61104 sera was as efficient as the anti-LeY-PAA sera in mediating CDC, while the anti-K61105, anti-K61106 and K61107 sera displayed a higher capacity for cytoxicity than the LeY-PAA form. Immunization of mice with MCF7 cells also results in a predominate IgM

response that leads to significant CDC mediation of the MCF-7 tumor line. No lysis was observed with pre-immune sera (<1:10), or with the anti-ganglisode antibody ME361. MCF7 is reactive with the anti-LeY monoclonal antibody BR55-2 to which BR55-2 was raised (3, 13, 14). These data indicate that serum generated to carbohydrate-mimicking peptides have the potential to recognize LeY and display a required functionality.

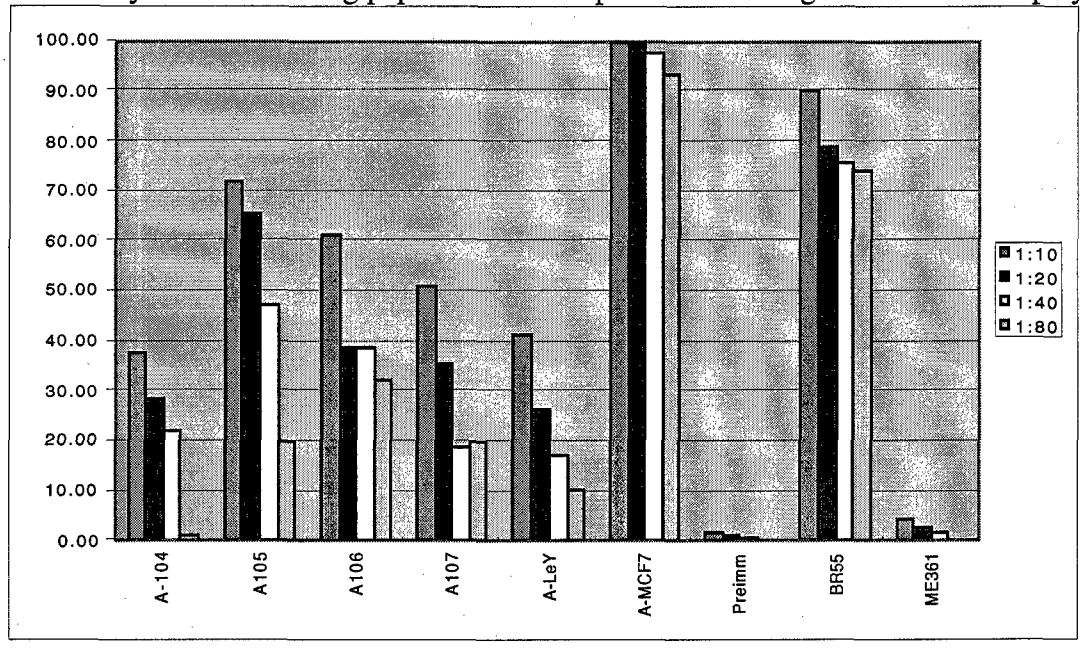
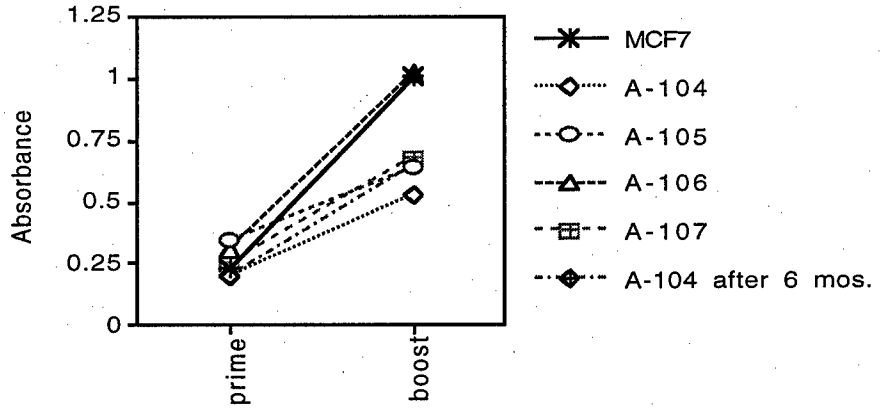


Figure 7. Titration of Complement-Dependent Cytotoxicity of MCF7 cells. Sequences of peptides 104, 105, 106 and 107 are shown in Table 3.

3.4 Induction of memory response

Peptide mimotopes might help elicit longer lasting immune repsonses to tumors providing immunological memory related to vaccine composition, form, and delivery. Memory responses could thwart repeated presentation of metastases as a consequence of maintenance of high levels of circulating antibodies. In studies relevant to understanding mechanisms associated with the utility of peptide mimotopes for vaccine efficacy, priming with mimicking peptides might establish beneficial memory responses for the induction of long-lasting carbohydrate cross-reactive IgM responses. It is observed that mice primed with the human breast tumor cell line MCF7 do not mount a substantial immune response after the primary immunization (Figure 8). A subsequent boost results in an enhanced anti-LeY immune response (Figure 8). To test if the peptides prime for an anti-LeY response, mice were immunized 3 times with a low dose (25ug) of the respective peptides with QS-21 as adjuvant, followed by boosting 1 time with MCF7 cells (without adjuvant) two weeks after the last peptide immunization. Immunization with peptides at this dose results in an anti-LeY response similar to that observed with primary immunization with MCF7 cells. Immunization with higher doses results in serum with higher LeY reactivity in ELISA assays (21, 25, 27). Upon subsequent boost with MCF7 cells, a two-fold increase in LeY reactivity is observed (Figure 8) for peptides 104,105 and 107. Priming with the WRY containing peptide 106 parallels the four-fold increase in the anti-LeY response observed with boosting of MCF7 primed mice with MCF7. The isotype was again predominately IgM in all cases. Response to LeY is also observed in mice primed with peptide 104 that have rested for 6 months and then boosted 1 time with the MCF7 line. LeY reactivity was the same as that observed for 105 and 107. The reciprocal experiment in which mice primed with MCF7 cells are boosted with peptide did not lead to enhanced anti-LeY serum reactivity (data not shown). Consequently, this data is suggestive that peptide mimotopes can prime for memory responses directed toward tumor cells expressing LeY.



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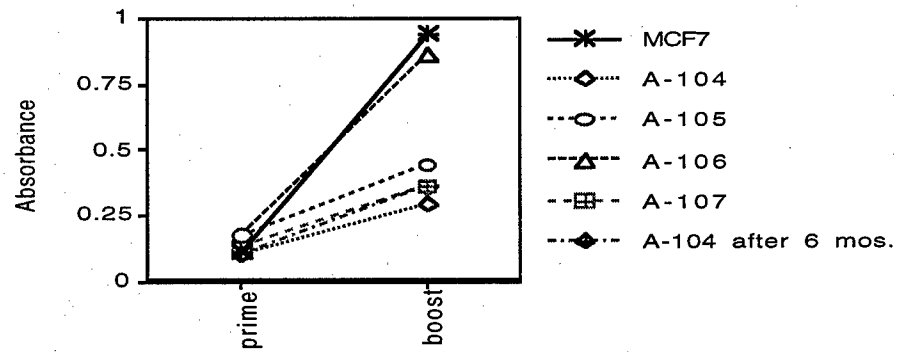

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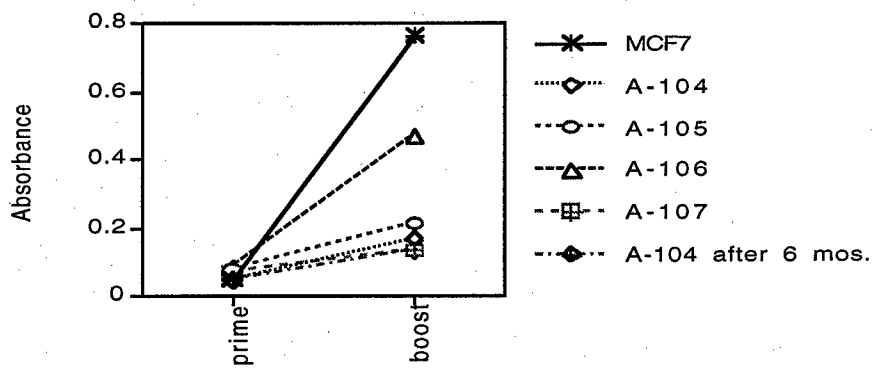

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Figure 8. Priming responses to peptide mimotopes. Peptides used in this study correspond to those shown in Table 1 synthesized as MAP peptides. No adjuvant was used with MCF7 cells. Serial dilution corresponsing to 1:50 (shown in A), 1:200 (B) and 1:800 (C) are shown.

3.5 Tumor Challenge

Further evidence for in vivo functionality of peptide mimetic vaccination comes from tumor challenged mice. LeY is not expressed in mice. Subsequently, a mouse model is not yet available to study the in vivo functionality of mice primed with peptides and then challenged with LeY expressing tumor. However, Balb/c mice do express sLeX. An anti-Id made against the monoclonal antibody FH6 has proved to be an effective mimic for sLeX, increasing the median mouse survival time of anti-Id immunized Balb/c mice after tumor challenge with the fibrosarcoma Meth A tumor cell line (64). A peptide with the sequence ISDGTTYTYYPDS derived from CDR2 of the heavy chain of the anti-Id appears to be responsible for a portion of this anti-tumor response (64). This anti-Id derived peptide displays homology with our aromatic peptides in being composed of Tyr residues and displaying homologous hydroxyl groups on the Thr residues. Consequently, we asked if our peptide mimotopes would produce a significant anti-tumor effect in Balb/c mice when challenged with Meth-A tumor cells.

We first examined the survival of groups of host mice immunized with peptide mimotopes compared with control immunizations, followed by challenge with sLeX expressing Meth A cells (Table 6). Ten days after the third immunization, mice were challenged subcutaniously (sc.) with 10^6 live Meth A cells (day 0). Survival times of host mice were monitored. The results in Table 6 indicate that preimmunization with mimicking peptide induced an immune response that prolonged survival time. The p value was <0.001 for the K61106 peptide when the generalized Wilcoxon test was performed on the differences between the survival of the group preimmunized with peptide and that of the group preimmunized with a control peptide, or that of the group receiving no treatment.

Table 6. Survival of groups of host mice preimmunized with peptide-proteosome conjugate compared with contr immunizations followed by challenge with sLeX expressing Meth-A cells.

Immunogen	Nominal antigen mimicked	Number of mice immunized	Survival Time (months)	Statistical significance compared to:	
				None	C1
1105	Le	12	2.44±1.23	p<0.05.	p<0.05
1106	Le	12	3.60±1.56	p<0.001	p<0.005
1107	Le	12	2.40±1.32	p<0.05	p<0.05
G1	GD2/GD3	11	1.45±0.33	N.S.	N.S.
QS-21	-	11	1.51±0.43	N.S	N.S.
Proteosome	-	11	1.48±0.42	N.S.	N.S.
C1	-	11	1.44±0.22	N.S.	-
None	-	12	1.46±0.26	-	N.S.

None is tumor alone. N.S. Not Statisticaly significant.. Proteosome is immunologic carrier control derived from meningococcal outer membrane proteins. The C1 peptide was coupled to the proteosome carrier formulation. G1is a control MAP peptide that mimics another tumor associated carbohydrate antigen.

The above studies were designed as a survival study using a high concentration of tumor cells (10^6) We subsequently initiated a kinetic study using a low, $5X10^4$, and moderate $3X10^5$ concentration of Meth A tumor cells. Mice were again immunized 3 times with mimicking peptides, followed by sc immunization with Meth A cells (Figure 9). The growth kinetics indicate that tumors grew slowly or not at all when peptide immunized mice were challenged with tumors within this dose range, suggesting that protection is dependent upon the concentration of tumor cells given to the animal. These data are further suggestive that peptide mimotopes of carbohydrates can prime for beneficial anti-tumor immune responses.

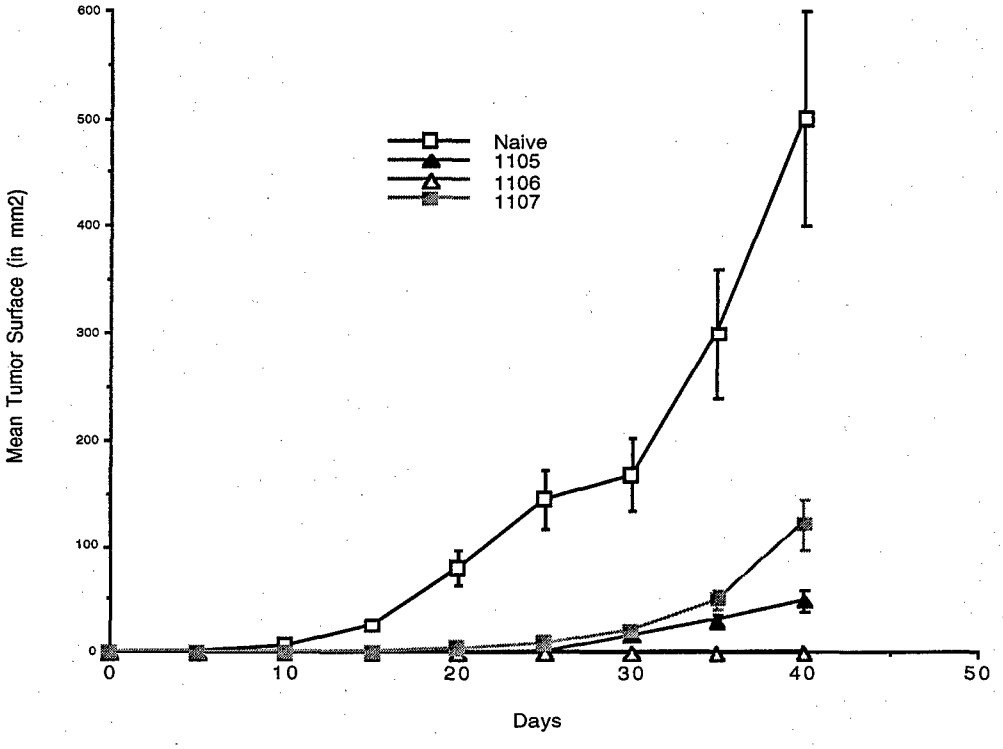

Figure 9. Tumor growth of mice preimmunized with peptide and challenged with sLeX expressing Meth-A tumor cells.

Conclusions and future directions:

The pattern of carbohydrate antigens expressed by different tumor types has been established, paving the way for polyvalent-antibody inducing vaccines (65, 66). The basis for carbohydrate based cancer vaccines that primarily induce an antibody response is well established in both experimental models and the clinical setting (10, 62, 65-77). It is the expectation, based upon evidence from carbohydrate vaccination trials (71, 72, 78-83), that antibodies can play a role in vivo in tumor regression, potentially opsonizing tumor cells to prevent extravasion, intravasion and metastatic potential. It is envisioned that antibody induction is especially effective in the adjuvant setting when the targets are circulating tumor cells and micrometastases (15, 84). In general terms, with regard to a major objective of carbohydrate vaccines--i.e., generation of cytotoxic antibodies--variability in patient response and lack of persistence of high-titered IgM cytotoxic antibodies in many patients immunized with carbohydrate-conjugates are problems that remain to be solved and emphasizes the still current limitation of this type of approach.

Molecular mimicry of carbohydrate antigens by peptide mimotopes is one way to augment immune responses to carbohydrate antigens in that they are intrinsically T cell-dependent antigens. We have shown that peptide mimics of neolactoseries antigens can induce IgM antibodies that cross-react with natively expressed LeY and sLeX antigens on the human tumor surface. It is often thought that IgM may not be therapeutically beneficial in spite of consistent documentation of the clinical benefit of anti-ganglioside IgM antibodies (10). This misconception is based on the immune response to protein antigens, which suggest that the IgM response is only transient and not persistent. IgM changes from "planar" to "staple" conformation when it binds to clustered epitopes. The "staple" conformation facilitates complement fixation and complement-mediated lysis. Serum containing predominately anti-LeY IgM is functional as measured by CDC in the presence of human complement, overcoming the known inefficenicy of homologous complement lysis. Using DNA vaccination approaches (28) we are examining the extent to which the peptide mimotopes can mediate T cell responses.

Engineered antibodies based on murine monoclonal antibodies directed against LeY will be generated for use in the diagnosis and therapy of breast tumors in patients. The use of murine monoclonal antibodies has been limited by their immunogenicity and lack of effector functions. Many of these shortcomings have been overcome by the use of genetic engineering to produce chimeric mouse/human antibodies. We have produced chimeric antibodies based onBR55-2, a high-affinity, highly specific anti-LeY antibody; these are available for therapy studies. During the upcoming year we propose to develop additional engineered antibodies that differ in key properties such as size, valency, and affinity, such that the effects of these characteristics on biodistribution and therapeutic index can be evaluated. Genetically engineered fragments (Fab, F(ab')2), single chain antibodies, CH2 deletion, and minibody will be developed during the next project period. Based on results on immune responses to radiolabeled antibodies, antibodies with reduced immunogenicity will be developed. Specific mutations will be introduced to permit site- specific chemical conjugation (ser to cys mutations) and glycosylation (asn-X-ser/thr), to facilitate coupling of chelate or protecting groups to modulate half-life and immunogenicity. Stably transfected cell lines producing engineered antibodies at high level will be generated. Bacterial expression will be used for single chain antibody and derivatives. These studies will contribute to the rational design of antibody/chelate/radiometal agents for use in radioimmunotherapy of Breast tumors (29).

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Manuscripts in Submission

1. A structural perspective of peptide mimotopes of a carbohydrate antigen

2. Vaccination with a carbohydrate peptide mimotope promotes anti-tumor responses.

Appendix Material

Manuscript #1. Murali R, Kieber-Emmons T. Molecular recognition of a peptide mimic of the Lewis Y antigen by an anti-Lewis Y antibody. Journal Molecular Recognition 1997. Vol. 10 269-276.

Manuscript #2. Luo, P., Qiu, J-P., Chang,T.Y., Steplewski, Z., Kieber-Emmons, T. A structural perspective of peptide mimotopes of a carbohydrate antigen. 1998. Submitted

Manuscript #3. Luo P, Agadjanyan M, Qiu J-P, Westerink MAJ, Steplewski Z, Kieber-Emmons T. Antigenic and immunological mimicry of peptide mimotopes of adenocarcinoma associated carbohydrate antigens. Molecular Immunology 1998;in press.

Manuscript #4. Kieber-Emmons T. Peptide mimotopes of carbohydrate antigens. Immunologic Research 1998;17:95-108.

Manuscript #5. Kieber-Emmons,T., Luo,P., Qiu, J-P., Chang, T.Y., Blaszczyk-Thurin, M., Steplewski , Z. Vaccination with a carbohydrate peptide mimotope promotes anti-tumor responses. 1998. Submitted.

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Molecular Recognition of a Peptide Mimic of the Lewis Y Antigen by an Anti-Lewis Y Antibody

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Peptides as mimics of carbohydrates display a distinct advantage in vaccine design because of ease of synthesis and their inherent T cell-dependent nature as immunogens. While peptides that mimic carbohydrates have been described, it is not clear how they do so. To further our insight into structural relationships between peptide-mimics and carbohydrate structures, we have analyzed a potential recognition scheme between the murine monoclonal antibody, B3, directed against the tumor-associated antigen Lewis Y oligosaccharide and a peptide identified from phage display screening with B3. The Lewis Y core antigen is a difucosylated structure consisting of four hexose units. The B3 antibody binds to the peptide sequence APWLYGPA in which the putative sequence APWLY is critical for binding to the antibody. Not having experimental structural information for B3, the crystal structure of another anti-Lewis Y antibody, BR96, solved in complex with a nonoate methyl ester Lewis Y tetrasaccharide, provides a molecular basis for LeY antigen recognition and specificity, and how this binding relates to peptide binding. As a guide to place the APWLY motif in the B3 combining site, a fragment library was searched for analogous compounds that have the potential to bind to B3. Our modeling study shows that the B3-peptide complex shares similar recognition features for the difucosylated type 2 lactoseries' structure. This analysis provides a molecular perspective for peptide mimicry of a carbohydrate epitope. © **1998 John Wiley & Sons, Ltd.**

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Introduction

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. The roles of carbohydrate structures present on the cell surface range from influencing tumor growth, progression and metastases, to mediating bacterial and viral attachment (Hakomori, 1989, 1991; Mandrell and Apicella, 1993). Subsequently, the inhibition of these interactions is a possible point of therapeutic intervention for a number of diseases. The definition of consensus carbohydrate structures on glycolipids or glycoproteins can serve as potential therapeutic targets (Tamatani *et al.*, 1995). Carbohydrates are, however, difficult to synthesize (Roy, 1996; Von and Colman, 1996) and are generally poorly immunogenic. Defining carbohydrate mimetics, or surrogate antigens, might provide an alternative approach to overcome such drawbacks.

One type of mimetic is peptides. Evidence for the ability of a peptide or polypeptide to mimic a carbohydrate determinant comes from several sources (Diakun and Matta, 1989; Sugiyama, *et al.*, 1991; Shikhman *et al.*, 1994; Tsuyuoka *et al.*, 1996). Anti-idiotypic antibodies have been defined that are mimics of carbohydrates, inducing immune responses that are cross-reactive with carbohydrate structures (Diakun and Matta, 1989; Chapman and Houghton, 1991; Furuya *et al.*, 1992; Cheung *et al.*, 1993; Saleh *et al.*, 1993; Westerink and Apicella, 1993). An appealing application for antibodies to identify carbohydrate surrogates, is the screening of peptide libraries with anti-carbohydrate antibodies. Screening against peptide display libraries identifies different molecular species than the one the antibody was raised against. Analysis of a larger repertoire of ligands reactive with an antibody combining site might establish structure/function relationships not evident from monoreactive molecules. Peptides that mimic carbohydrate structures have been defined in this manner (Oldenburg *et al.*, 1992; Scott *et al.*, 1992; Hoess *et al.*, 1993). Peptides reflective of those found from screening libraries can induce immune responses cross-reactive with carbohydrate structures (Westerink *et al.*, 1995; Hutchings *et al.*, 1996; Agadjanyan *et al.*, 1997; Kieber-Emmons *et al.*, 1997). These studies indicate that although antigenic mimicry of anti-idiotypic antibodies, or peptides, are accomplished using amino acids in place of sugars, the specificity pattern can be precisely reproduced.

The molecular basis for peptide mimicry of carbohydrate structures is not well characterized. For peptides as immunogens, it is important to distinguish the difference between chemical or antigenic mimicry, in which chemical similarity exists between the carbohydrate and peptide, and

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biological or immunological mimicry in which the mimetic induces particular carbohydrate reactive antibody subsets. In our efforts to elucidate how peptides might effectively mimic carbohydrate forms, we report here a strucural basis for antigenic mimicry of a peptide surrogate for the histo-blood group antigen Lewis Y (LeY). The LeY difucosylated type 2 lactoseries structure, Fucα1→2 Galβ1→(Fucα1→3)GlcNacβ→R, expressed on both glycoproteins and glycolipids, is one tumor-associated carbohydrate structure being explored as a target for monoclonal antibody (MAb) based imaging and therapy (Garrigues *et al.*, 1993; Choe *et al.*, 1994; Kitamura *et al.*, 1994; Co *et al.*, 1996; Yin *et al.*, 1996) and for vaccine development (Kitamura *et al.*, 1994). Lewis Y is poorly immunogenic in humans. A peptide with the putative sequence APWLYGPA, as identified by phage display screening with the anti-LeY antibody B3, competes with the LeY antigen for B3 binding (Hoess *et al.*, 1993) and this and related peptides might prove to be effective in enhancing the therapeutic utility of a LeY-conjugate vaccine.

The B3 antibody displays homology with other anti-LeY antibodies (Thurin-Blaszcyk *et al.*, 1996), including the recently reported structure of antibody BR96, co-crystallized with a nonoate methyl ester LeY tetrasaccharide (Jeffrey *et al.*, 1995). The crystal structure of BR96 provides an avenue to elucidate possible recognition relationships among Lewis Y reactive antibodies. Molecular modeling of B3 complexed with the putative tetrasaccharide core of LeY was performed based upon the BR96-sugar recognition scheme (Jeffrey *et al.*, 1995). The B3 model emphasizes key polar and nonpolar interactions contributing to the molecular recognition feature for LeY shared among related anti-LeY antibodies, and consistent with mapping profiles of lactoseries derivatives reactive with B3 (Pastan *et al.*, 1991).

While current procedures for predicting ligand–antibody interactions are limited, mainly due to the conformational flexibility of ligands and antibodies; and the role of solvent in mediating ligand recognition and binding, the utilization of a crystallographically determined starting position can, nevertheless, lend to discriminating differences in binding orientations of analogs. Using the positioned LeY structure in B3, we implemented the program Ligand-Design (LUDI (Bohm, 1992) Biosym Technologies) to search a fragment library to guide in the position of the putative APWLY peptide sequence. Optimization of the positioned peptide indicates some preferences for B3 contact sites. This analysis therefore provides a unique perspective of how peptides, and perhaps anti-idiotypic antibodies, are cross-reactive with V domains specific for carbohydrate antigens.

Experimental

Model building and energy refinement

The B3 structure was developed based upon the BR96 crystal structure 1CLY(Jeffrey *et al.*, 1995). The CDRs and the framework (FR) of the crystal structure template were mutated to those of the respective B3 heavy and light chains using Insight II. The side chain angles of the substituted residues were set according to angles identified in a database of side chains. Each CDR and framework region was changed individually, followed by 1000 cycles of energy minimization to eliminate close contacts between atoms. As in our previous studies, the program Discover (version 2.95 Biosym Technologies) was used for conformational calculations with the supplied consistent valence force field (CVFF) parameters. After model building, the respective structure was energy optimized to convergence. Molecular dynamics (MD) at 300 and 600 K was used to further alleviate any close contacts during model building.

Initially a molecular dynamics simulation over 30 ps using the program Discover was performed. The structure was then energy minimized using conjugate gradients to convergence. Following this initial equilibration, the calculation was resumed for another 30 ps at 600 K at constant pressure and then cooled to 300 K over 50 ps. During the second dynamics procedure, atoms lying further than 15 Å from all atoms of the CDR loops were held fixed. Non-hydrogen atoms of residues lying in the region 9 to 15 Å from all CDR loop atoms were harmonically restrained to their initial positions with a force constant of 30 kcal × mol^{-1} × rad^{-2}. These distance approximations result in fixing or restraining atoms of residues within the framework region of the antibodies. The backbone conformation torsion angles, phi and psi, of non-CDR loop residues were restrained to their initial values with a force constant of 1600 kcal × mol^{-1} × rad^{-2}. In addition, a torsional restraint of 10 kcal × mol^{-1} × rad^{-2} was employed around the omega bond. A time step of 1 fs was used. The resulting structure for B3 was again energy minimized using conjugate gradients to convergence.

Docking of Lewis Y to B3

The approach taken in the placement of the Lewis Y core in the antibody combining site made use of the position established from the BR96 crystal structure. After minimization, a molecular dynamics calculation over 100 ps using the program Discover was performed. Restraints were imposed to enhance the idealized hydrogen bonding pattern suggested from the heavy atom distances observed in the 2.6 Å map of BR96 (Jeffrey *et al.*, 1995). The dynamics run was not intended to be a detailed study, but to further alleviate any close contacts within the antibody and between the tetrasaccharide and the antibody. The calculation was initialized and equilibrated for 100 ps at 300 K at constant pressure and resumed for another 50 ps. The resulting structure was energy minimized using conjugate gradients to convergence. Charges and non-bonded parameters for the LeY structure were assigned from atom types from the CVFF parameter list supplied with Discover/InsightII.

Peptide placement within the B3 combining site

A LUDI search was performed using standard default values and fragment library supplied with the program to identify fragment positions within the B3 binding site. This program constructs possible new ligands for a given protein of known three-dimensional structure. Small fragments are identified in a database and are docked into the protein binding site in such a way that hydrogen bonds and ionic interactions can

be formed with the protein and hydrophobic pockets are filled with lipophilic groups of the ligand. The positioning of the small fragments is based upon rules about energetically favorable non-bonded contact geometry's between functional groups of the protein and the ligand. The center of search was defined using the crystallographic LeY position. In this approach the OH-3^c position on the LeY structure was used as the sampling point. To identify possible fragments that are similar to the putative APWLY sequence, the radius of interaction, which defines the size of spheres in which LUDI is to fit appropriate fragments, was set as incrementing radii from 5 to 14 Å . Results of the search were compared with the putative peptide sequence side-chain types, with those LUDI fragments retained within the B3-Lewis Y binding site which displayed similarities with the putative peptide side-chains. The peptide was built using InsightII and positioned relative to the docked LUDI fragments. The peptide backbone and side chain torsional angles were rotated until the side-chains of the peptide were approximate to the corresponding LUDI fragments. The peptide-B3 complex was subjected to energy optimization and molecular dynamics simulations as with the LeY-B3 complex.

Results

Structural properties of the BR96 template

The crystal structure of BR96 provides a template to directly model the homologous B3 structure. B3, shares a high degree of sequence homology with BR96 and another anti-LeY antibody BR55-2 in both their light and heavy chains (Fig. 1). The primary structure of the light chain of the anti-cholera toxin antibody TE33 (ITET) and the autoantibody BV04-01 (1CBV) display 92 and 87% identity, respectively, with the BR96 light chain (Fig. 1). Both of these antibodies have been elucidated by X-ray crystallography as a complex with their respective ligands. Superposition of these two light chains with BR96 indicate that their CDR conformations are nearly the same except for CDR1 around the sequence tract 'S-N-G' of BV04-01 which is homologous

Figure 1. Sequence alignment of variable region Light and Heavy chain s of B3 with crystal template structures. Sequences for BR96, TE33, BV04, and BI3i2 are from deposited PDB files in the Brookhaven repository (Berstein *et al.*, 1977). Sequence for B3 is from (Choe *et al.*, 1994) and BR55-2 from (Thurin-Blaszczyk *et al.* 1996). Numbering corresponds to that of the BR96 crystal structure. Respective CDRs are underlined for each chain. LeY contact residues that are conserved between the anti-LeY antibodies BR96, and B3 are in Bold face on the BR96 sequence.

with B3; RMS differences are 0.63 and 0.55 for BV04-01 and TE33, respectively, excluding the CDRI region. The conformational differences are either the result of sequence differences or due to induced conformations upon binding of the respective ligands. Crystal analysis of unligated BR96 suggests that CDRIL of BR96 undergoes a transition upon LeY binding (Sheriff *et al.*, 1996).

For the heavy chain of B3, the IgGl Fab' fragment Bl3i2 (2IGF) co-complexed with a myohemerythrin derived peptide, displays 77% identity with both BR96 and B3, having the same length CDR3. Superpositioning of these structures indicate that they display nearly the same conformations up to the CDR3 region (residues 1–92) with an RMS of 0.53 Å, suggesting CDR1 and CDR2 conformations have undergone similar conformational transitions if any. While the CDR3 length found in Bl3i2 is the same as that for BR96, not unexpectedly the conformations are different in the two crystal structures. The CDR3 loop in BR96 is typical of a type II turn with a Gly in the *i*th + 2 position with the two Asp residues at positions 97 and 98 pointing outward from the antibody combining site towards solvent. Mutatin of the native aspartic acid at position 97 in BR96 to alanine, results in increased tumor cell binding of BR96 (Yelton *et al.*, 1995). Interestingly, Ala is the native residue at this position in B3. Analysis of the nonoate methyl ester moiety of the complexed LeY tetrasaccharide suggest that this Ala could interact with a sugar residue extending from trifucosylated structures extended at the reducing site (our unpublished observation). This is consistent with the observation with another anti-LeY antibody AH-6 which binds equally well to LeY hexaosylsceramide, difucosylated LeY octaosylceramide and trifucosylated nonaosylceramide suggesting that the epitope is limited to the LeY hexasaccharide or the extension at the reducing part of the oligosaccharide is incorporated into the binding groove without influencing antibody binding.

The BR96 based model was mutated to the B3 sequence and energy minimized. A relatively short dynamics run was performed to relieve any short contacts in the modeled structures. Molecular dynamics studies on antibodies indicate that major transitions in torsional angles are observed during the initial equilibration stage (Tanner *et al.*, 1992). It was not the intent to perform a detailed study of possible transitions of the CDR loops since in the "homology" modeled structure, hydrogen bonding constraints were to be invoked to optimize the suggested interactions defining the binding mode of the sugar moieties determined in the BR96-LeY complex. In general, structures of free and antigen bound antibodies demonstrate the flexibility of the antibody combining site and provide an example of induced fit as a mechanism for antibody-antigen recognition (Rini *et al.*, 1992). Transitional differences are in fact observed for CDR1L, CDR3L and CDR2H of BR96 comparing ligated and unligated structures (Sheriff *et al.*, 1996). Dynamic transitions within CDRs of unligated antibody forms have been suggested to be irrelevant to explain such induced fit binding mode geometry's (Rini *et al.*, 1992). We observe that using homologous templates of antibodies that are co-complexed account for the observed transitions in CDR2L and CDR2H for BR96. Interestingly, CDR3H shows no difference in conformation between ligated and unligated BR96 (Sheriff *et al.*, 1996). This might be because of the turn type and stabilizing effects contributed by Trp 100a interacting with a backbone carbonyl oxygen (Sheriff *et al.*, 1996).

Placement of the Lewis Y tetrasaccharide core antigen in the B3 binding site

Suggested hydrogen bonding sites between BR96 and the tetrasaccharide core structure of LeY (Jeffrey *et al.*, 1995) are observed to be highly conserved between BR96 and B3 (Fig. 1). Suggested contacts are based upon heavy atom distances measured in the BR96 structure (Jeffrey *et al.*, 1995). Further analysis of these distances indicate that some of the suggested hydrogen bonding schemes are far from a geometry to form hydrogen bonds when considering the orientation of added hydrogens on donor–acceptor pairs. To identify the most likely set, the complexed BR96 based B3 structure was energy optimized with and without suggested hydrogen bonds as restraints followed by 100 ps of molecular dynamics. In a first set of calculations, no restraints were invoked (Fig 2a). In this calculation Tyr H35 forms a hydrogen bond with OH-6^{b}, Ser L27E forms a hydrogen bond with OH-4^{d} and the backbone amide group of Ala H100 interacts with OH-4^{c}.

In a restrained calculation, the backbone amide group of Ala H100 was forced to form a potential hydrogen bond with OH-4^{c}, along with a hydrogen bond between His L27D and OH-3^{b}, and between O7^{a} and the backbone NH group of Tyr H33. In this placement, the backbone amide of Leu H96 also interacts with OH-2^{c}. Intermolecular energy calculations between LeY and B3 indicate that the configuration in Fig 2b is 3 kcal more stable than that of Fig 2a. Imposition of the constraints in Fig 2b results in an RMS of 0.5 Å in the Cα positions relative to the conformation in Fig 2a. This analysis indicates that restraints are needed to fully realize idealized hydrogen bonding geometry's but the imposition of these restraints does not alter significantly the overall conformation of the antibody template.

Peptide placement within the B3 combining site

In the placement of the B3 reactive putative peptide sequence APWLY, we made use of the program LUDI to identify compounds that potentially interact with the B3 combining site. Over 260 fragments were identified for the model, with the largest radius of interaction, with most redundant for the same set of potential hydrogen bond donors or acceptors on B3. In evaluating the fragments we compared fragments identified by LUDI relative to the APWLY sequence such that the fragments could occupy non-redundant sites and be spatially far enough from each other to accommodate the peptide backbone. In Plate 1 the placement of representative LUDI fragments is shown relative to their positions with each other within the B3 binding site of the respective models. LUDI found that a Trp like residue forms a hydrogen bond with the backbone carbonyl oxygen of Trp H98, that a lipophilic residue representative of a Leu side chain is bounded by residues Val L94, His L27D, and Ala H58 another lipophilic residue representative of an Ala and Pro side chain is bounded by

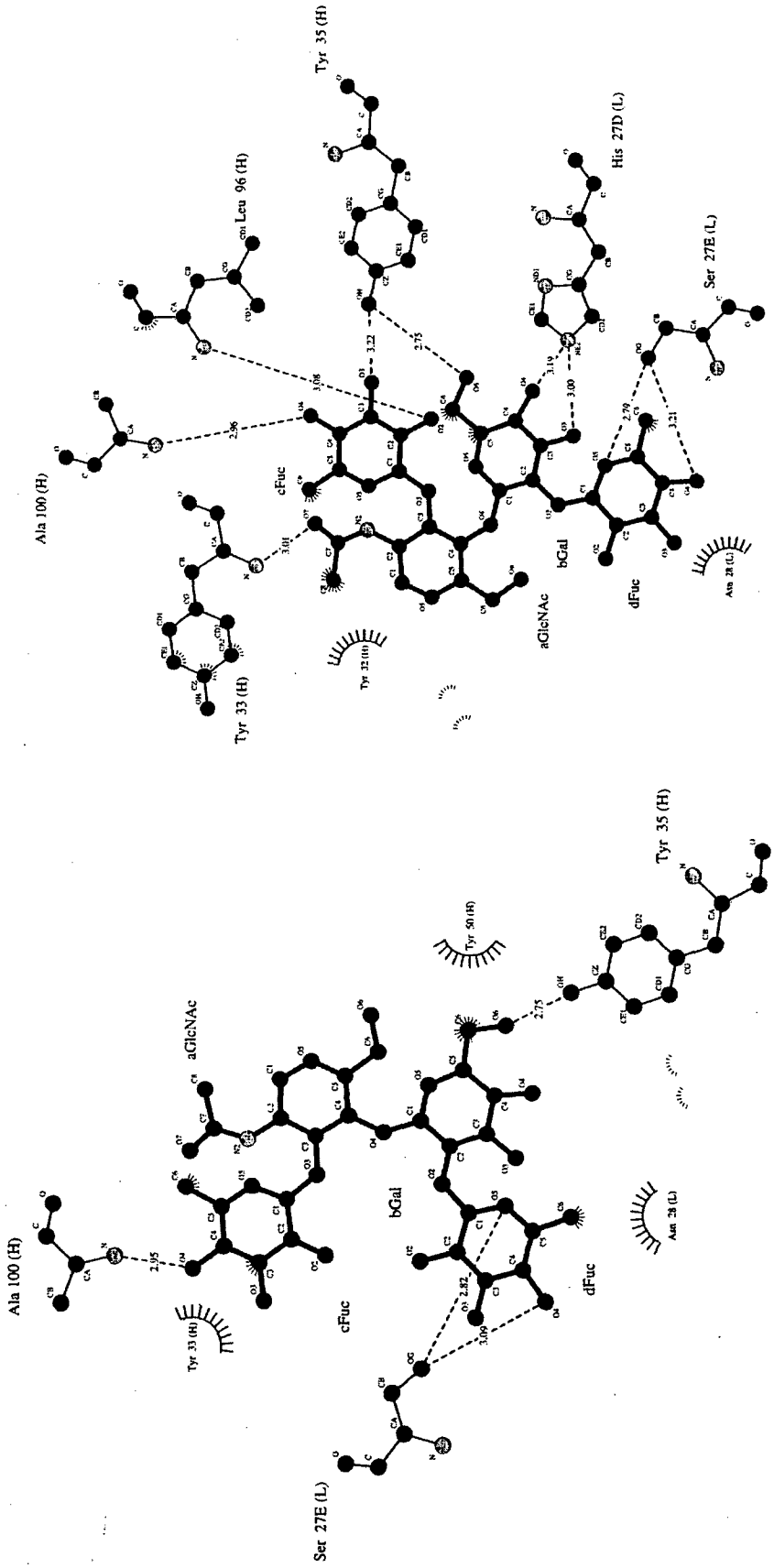

Figure 2. Hydrogen bond interaction schemes of B3 with the LeY tetrasaccharide core under different constraint conditions. The position of LeY is that observed in the BR96 crystal structure. The a, b, c, d designations refer to the βDGlcNac, βDGal, αLFuc(1→3) and αLFuc(1→2) units, respectively. Figures 2a and 2b are interaction schemes for the BR96 based model. Intermolecular interaction energies are − 73 Kcal (Fig 2a), and − 76 Kcal (Fig. 2b). Drawings were made with LIGPLOT (Wallace *et al.*, 1995).

Ala H97 (Plate 1).

The APWLY sequence was then modeled; such that the corresponding Trp, Pro, Leu and Ala residues occupied relative positions as the identified LUDI fragments (Plate 2). In affect one wants to 'stitch' the fragments together to form a peptide. We modeled the peptides two ways. The first, was to use individual amino acid fragments oriented with their side chains superimposed on the LUDI identified side chain types. The individual fragments were then restrained to form concomitant backbone geometry's and conformations. As expected, such an approach resulted in highly strained conformations. Alternatively, a peptide was built and the phi, psi angles rotated until the respective side chains were in close proximity. The positioned peptide fragment-B3 complex was then energy optimized with a restrained dynamics calculation. After this dynamics run, the complex was again energy optimized to convergence without the imposition of constraints. Deviation of the backbone conformatin of the peptide-B3 complex relative to the respective LeY-B3 complex was found to be only 0.29 Å. This indicates that the placement of the peptide within the antibody combining site did not dramatically alter the overall conformation of the B3 structure.

While the LUDI search provided a favorable geometry for peptide side chain placement, the final placement of the peptide side-chains within the antibody combining site relative to the LUDI positioned fragments were different (Plate 2). Several different starting geometry's for the peptide placement in the BR96 model were tested. Intermolecular interaction calculations indicate that the majority of the peptide binding comes from dispersion interactions. Five potential hydrogen bonds were found for the most stable of the models (Plate 3). One involves the N7 of Trp interacting with the backbone carbonyl group of Trp H98, the carbonyl backbone of Trp interacting with His L27D, the Tyr side-chain hydroxyl group interacting with hydroxyl group of Ser H55, the backbone carbonyl group of Ala interacting with Asn H52A, and Tyr H33 side-chain interacting with the carbonyl backbone of Leu, whose hydrophobic side chain being further stabilized by dispersion interactions with Val L94. We have further constrained the model peptide to form a beta turn in which a hydrogen bond is potentially formed between Tyr amide and Pro carbonyl groups.

In this positioning we observed that the Ala-Pro residues of the peptide occupied a similar position as the LeY GlcNAc residue. This positioning indicates that the proline residue mimics the spatial position of the glucose unit of GlcNAc, while the Ala methyl group is positioned similarly as the terminal methyl group of GlcNAc's *N*-acetyl (Plate 4). The Trp residue occupies a volume associated with the cFuc residue, and the Leu residue occupying the volume and the hydrophobic interaction of bGal. The Tyr residue occupies a position not associated with the LeY binding to B3.

Discussion

We have developed peptide mimics of carbohydrates that induce humoral immune responses reactive with bacteria, virus and tumor cells (Kieber-Emmons *et al.,* 1996; Hutchings *et al.,* 1996; Agadjanyand *et al.,* 1997; Kieber-Emmons *et al.,* 1997). This approach provides one more strategy that makes use of general immunological principles. In particular, peptides that mimic carbohydrates might be used to augment naturally available immunoglobulins to tumor antigens or induce memory responses to carbohydrates in a combined peptide/carbohydrate-conjugate vaccine approach for immunotherapy. Carbohydrate antigens by themselves are poorly immunogenic, difficult to synthesize and are important targets for immune attack. As demonstrated in our previous studies (Westerink *et al.,* 1995; Hutchings *et al.,* 1996; Agadjanyand *et al.,* 1997; Kieber-Emmons *et al.,* 1997), appropriately constructed peptides may indeed be able to augment immunogenicity against carbohydrate antigens. The approaches to identifying these peptides include the use of phage combinatorial libraries for construction of a great range of peptides which are selected using carbohydrate reactive antibodies. From a molecular perspective, screening a phage display peptide library might identify a population of peptides reactive only with the isolating antibody. These peptides might, nevertheless, mimic salient features of a carbohydrate antigen reactive with a parallel set of carbohydrate specific antibodies. Modeling of isolated peptide and peptide monoclonal antibody conformations might suggest sites of amino acid changes in the peptides for augmenting antibody specificity or designing peptides that induce a broad range of carbohydrate reactive antibody subsets.

As a model system, we examined the molecular basis for reactivity of a peptide that mimics the tetrasaccharide core of the Lewis Y antigen, isolated from phage display screening (Hoess *et al.,* 1993). Based upon the crystal structure of the BR96-LeY tetrasaccharide complex and the relative sequence similarities between anti-LeY antibodies (Fig. 1), it is apparent that the MAb binding groove of LeY specific MAbs is sufficiently large to bind four monosaccaride units of the LeY determinant (Fig. 2) and fit a putative peptide surrogate that effectively mimics LeY binding. The similarities among the anti-LeY antibodies provides a restraint on the binding site configuration for LeY. The modeling of B3 reflects this point. The imposed binding mode restraint provided from the BR96-cocomplex provides limits as to the conformational transitions the CDR domains will undergo. It is well known that most changes in CDRs occur in the first 100 ps of molecular dynamics runs (Tanner *et al.,* 1992). However, the imposition of long dynamics run on unligated or uncomplexed structures do not represent potential binding mode geometry's for an antibody (Rini *et al.,* 1992). Structural analysis of unligated BR96 indicates that L1, L2 and H2 loops undergo transitions upon antibody binding, while H3 does not (Sheriff *et al.,* 1996). In our comparison we show that the conformational transitions observed for L1, L2 and H2 are those observed in other ligated antibodies. In effect this observation validates a rationale of using antibody templates that have undergone antigen binding to approximately model a transition state conformation for a ligated antibody form. In our modeling of B3, we so heavily constrain the movement of the B3 template backbone that only the side-chains adjust. In this way we are preserving the crystal structure determined binding mode geometry for BR96 as adopted by B3. Subsequently, for antibodies, it is not at all clear why

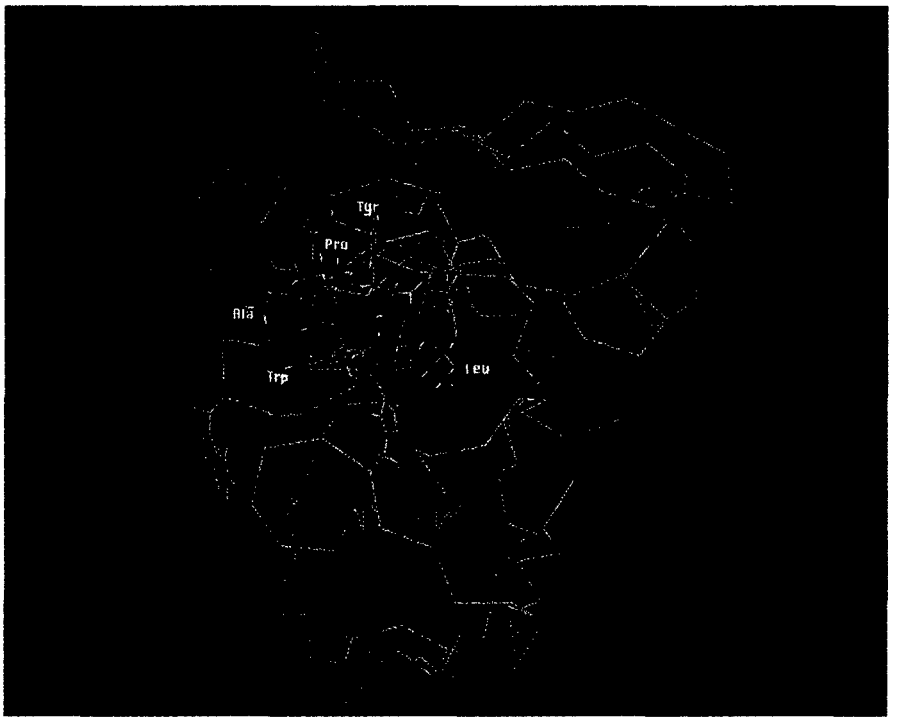

Plate 3. Low energy placement of the APWLY motif within the B3 combining site. The APWLY sequence is coloured green, highlighting nitrogens and oxygens colored blue and red, respectively, with hydrogens colored white. The B3 antibody is colored orange. The N terminus of the APWLY peptide is colored magenta and its C terminus is colored yellow. Both termini are pointing outward from the combining site, simulating a required orientation of the ends for extended residues. Hydrogen bonds are illustrated to respective B3 amino acids in the combining site.

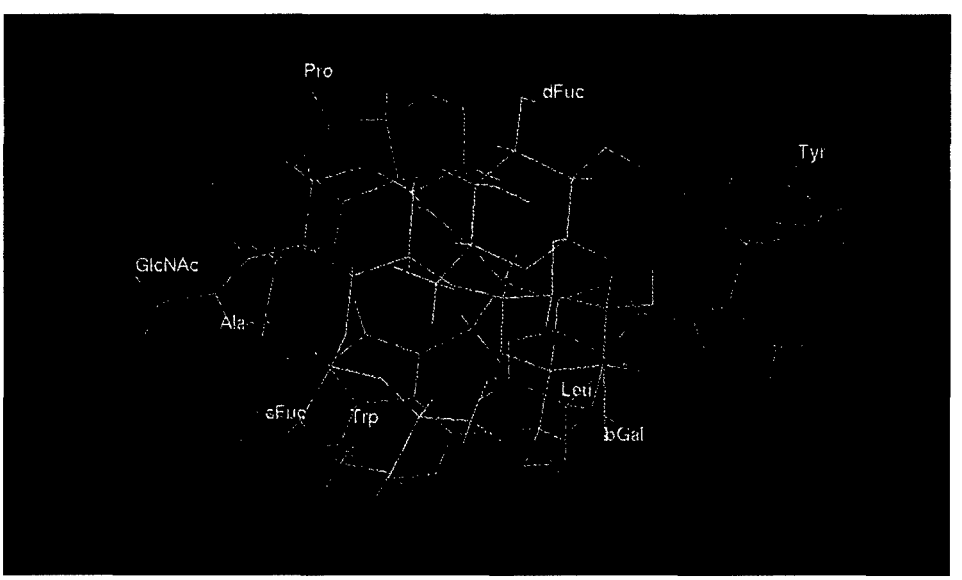

Plate 4. Overlap of the putative peptide with the LeY tetrasaccharide core. The peptide and the LeY structure are those of the binding mode conformation within the BR96 template derived combining site. In this orientation of the peptide within the site the Ala and Pro residues of the putative peptide sequence are observed to be spatially similar to the GlcNAc residue of the LeY structure.

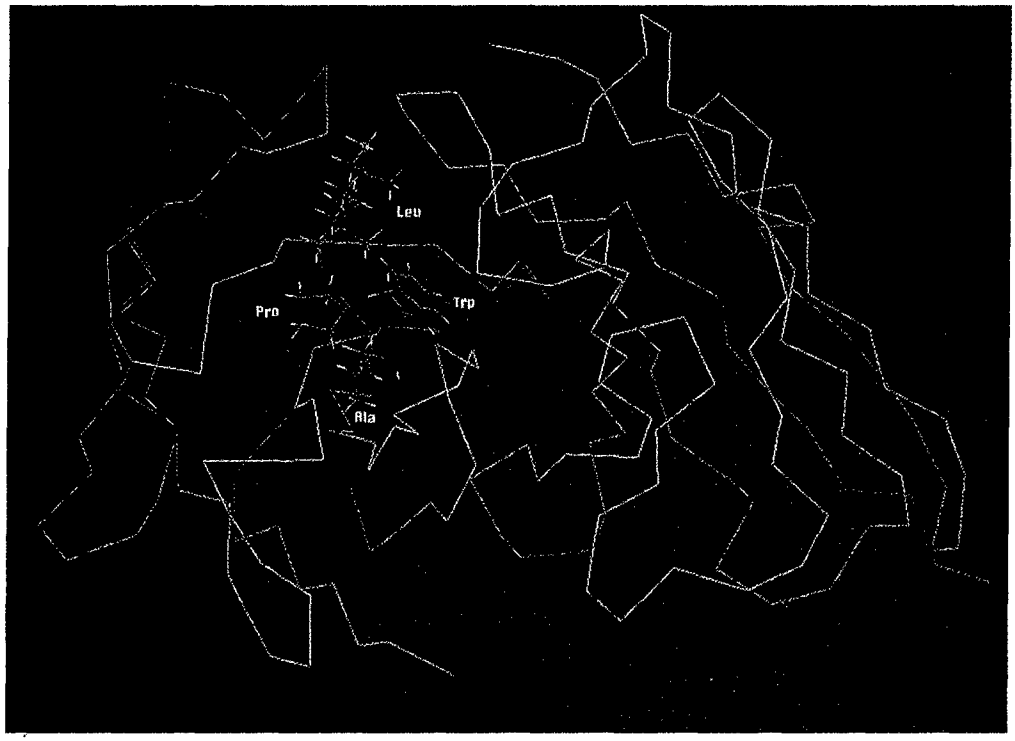

Plate 1. Selected LUDI fragments identified as representative amino acid side-chains comprising the APWLY motif. B3 is colored yellow in the BR96 based model. Selected fragments represent Ala, Pro, Trp, and Leu side chains.

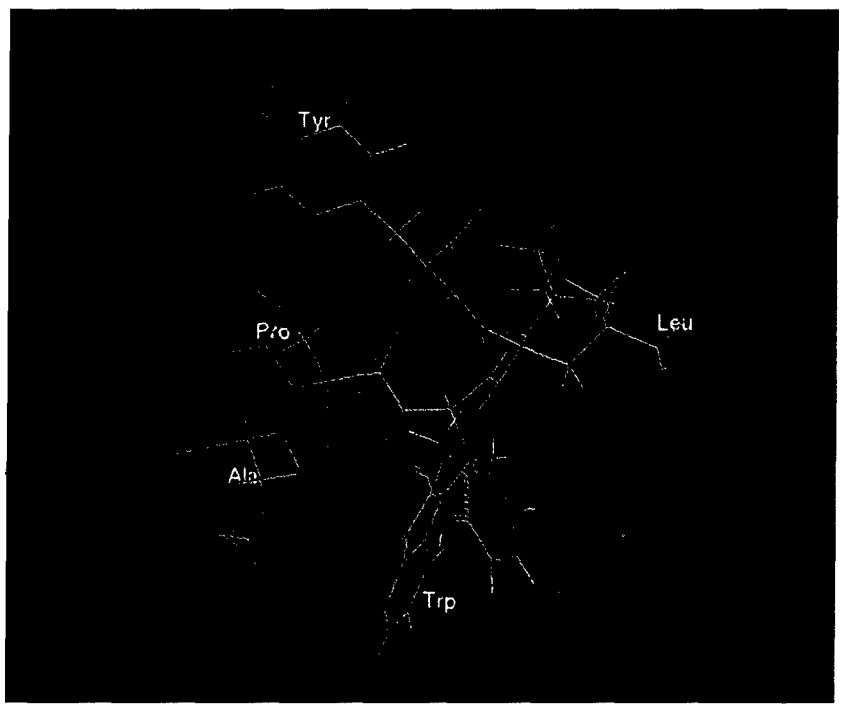

Plate 2. Fitting of the putative peptide sequence onto the LUDI defined fragments. The conformation of the APWLY peptide (colored cyan) is energy optimized binding mode in the B3 - BR96 template derived combining site. The Pro like fragment is colored magenta, the Trp like fragment is colored green and the Leu like and Ala liphophilic groups are colored orange.

MD calculations should ever be performed on unligated structures when one is interested in ligated conformations.

Structural studies on Lewis antigens have generally substantiated that conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles. Molecular dynamics calculations on Lewis antigen structure prototypes indicate the lack of spontaneous conformational transitions to other minima during the simulations, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes (Thurin-Blaszczyk *et al.*, 1996; Mukhopadhyay, and Bush, 1991). These results further indicate that hard shpere or rigid-geometry calculations, albeit in the absence of solvent, provides a good picture of the steric repulsion that modulate the conformational properties of the Lewis antigens. Subsequently the notion of utilizing a structure based drug design approach (Bohm, 1992) to determine a possible binding mode of a carbohydrate surrogate peptide offers a novel approach to confirm the ability of certain peptide residues to participate in contacting a receptor.

The search of a fragment library for possible compounds that would fit within the B3 combining site provides a guide to position the side-chains of the putative peptide sequence APWLY to effectively compete with the LeY antigen (Plate 1). The binding mode of the peptide did not faithfully mimic the LeY antigen in contacting all the same functional groups on B3, but binds in a fashion that provides for at least steric competition between the peptide and the LeY structure (Plate 3). We observe that the peptide might be availed to form a beta turn in the binding site. This conformation lends itself to the Tyr residue of the peptide to potentially interact with several residues in CDR2 of the heavy chain of B3 which include Asp H53, Ser H52, Ser H55 or Ser H56. These residues are different with respect to BR55-2 which does not effectively bind the APWLYGPA peptide (unpublished observations). It is noted that the positioning of the peptide within the B3 combining site is strictly a model and awaits confirmation from crystallographic studies on related anti-LeY reactive antibody-peptide complexes. Nevertheless, the model does suggest that peptides that contain the APWLY motif should bind to B3, effectively mimicking the LeY antigen as observed experimentally. The extent of potential fragments that we have found to interact with the B3 combining site indicates that there may still be many ways for peptides with differing sequences to interact with B3 in spite that only one peptide sequence was identified in the phage screening (Hoess *et al.*, 1993). In related studies, we have identified many peptides reactive with BR55-2 from phage display screening with BR55-2 (unpublished). One peptide within this set displays an inverted WPYL sequence compared with PWLY. While it is likely that peptides identified by screening phage libraries might only be reactive with an isolating antibody, the approach described here might help to identify potential peptides for immunization studies from the many peptide sequences identified from phage display screening to induce a broadly reactive antibody subset. In particular, further structural studies of histo–blood group related antigen binding to specific antibodies can provide information relevant to vaccine design strategies and improved immunotherapeutics for a variety of human cancers overexpressing these and related carbohydrate determinants.

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A Structural Perspective of Peptide Mimotopes of a Carbohydrate Antigen

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Abstract

Peptides may substitute for carbohydrates in reactions with carbohydrate-specific molecules. Using the recognition properties of an anti-Lewis Y antibody, BR55-2, as a model system, we establish a molecular perspective for such mimicry by comparing the three-dimensional basis for Lewis Y binding to the BR55-2 combining site with peptides that mimic Lewis Y in binding to BR55-2. The fine specificity of BR55-2 for peptides was evaluated by using the computer program LUDI to "epitope map" the BR55-2 combining site, identifing amino acids interacting with the same BR55-2 residue functional groups as the Fucα(1-3) moiety of LeY. The computer based screening approach was further used to define the potential mimicry of a peptide containing the putative sequence tract, FSLLW, identified as a potential mimotope of Lewis Y from screening BR55-2 with a peptide library. Molecular modeling and conformational analysis indicates that the FSLLW sequence adopts a type II β-turn within the BR55-2 combining site that serves to place the motif into a depression in the antibody combining site. Peptide binding is associated with only minor changes in BR55-2 relative to the BR55-2-Lewis Y complex. This peptide competes with LeY for BR55-2 binding and induces a LeY specific immune response. Fine specificity analysis also indicates that BR55-2 binds to peptides containing a W/YRY motif. These peptides too compete with Lewis Y for a shared binding site within BR55-2. Substituion of a Pro residue for Arg within the motif diminishes BR55-2 binding, indicating that aromatic residues alone are not sufficient for antibody recognition. These results further confirm that peptides and carbohydrates can bind to the same antibody binding site and that peptides can structurally mimic salient features of carbohydrate epitopes. A more precise understanding of the binding of peptide mimotopes at the molecular level is relevant for defining structural/function correlates necessary in vaccine and in inhibitor design applications.

Introduction

Carbohydrate antigens are important distinguishing landmarks on many infectious agents and neoplasms, and thus potential targets for preventative vaccination protocols. Unfortunately, carbohydrate vaccines are often poorly immunogenic, and induce weak IgM responses without long lasting immunity. It is known that some short peptides can cross-react with anti-carbohydrate antibodies, suggesting an alternative strategy for inducing a highly-directed, high affinity and specific anti-carbohydrate antibody response [1-4]. Because the choice of peptide is not obvious, we and others have previously reported the use of combinatorial methods to select peptidomimetics [5-20]. Due to the various limitations to these experimental approaches, and since the primary structures of anti-carbohydrate antibodies are frequently known, it would be ideal to be able to synthetically derive peptidomimetics from a molecular understanding of the binding site of these antibodies.

Pioneering work from several groups has clearly demonstrated that antigens of different chemical structures, such as carbohydrates and peptides, or nucleic acids and peptides, can efficiently bind to a single monoclonal antibody [5-20]. These findings have profoundly expanded the definition of molecular mimicry beyond simple chemical relatedness to include the more generalized notion of molecular relatedness. Heterologous binding by chemically-unrelated molecular surfaces may be a common phenomenon in many antigen-antibody interactions [21].

The molecular nature of mimicry between carbohydrates and peptides is still not well understood. Studies on carbohydrate mimicking peptides and their cognate antibodies suggest that aromatic-aromatic and hydrophobic interactions are critical chemical forces which modulate binding[1-3, 6, 13, 15-17]. Peptide mimotopes for carbohydrates have been defined containing a two aromatic amino acid repeat motif W/YXY found to bind to Con A (YPY) [6, 15], in peptides that mimic the Lewis Y antigen (WLY) [13], in peptides that bind to anti-Crytococcous polysaccharide antibodies [16] and that mimic the major C polysaccharide of *N. Meningitis* [3]. As previously noted [16], these observations suggest that a particular peptide structure is required for polysaccharide mimicry [22]. The role played by the aromatic rings might be to position particular residues for reactivity or they may directly bind to the antibody [13]. We and others showed that

peptide mimotopes either containing the W/YXY sequence tract or are homologues of the motif can adopt β turns in the antibody combining site [3, 23-25].

To better understand these interactions, we have been studying the structural basis of Lewis Y (LeY) binding to antibodies [24, 26]. LeY is a histo-blood group antigen found on Human Immunodeficincy Virus (HIV) [4] and Helicobacter pylori [27] and some human neoplasms, including certain breast, ovarian and prostate cancers [28]. Vaccinations with LeY-containing preparations can result in poor cellular and humoral immune responses. In addition, the immune response demonstrates various degrees of cross-reactivity with the related neolactoseries Lewis X (LeX), sialyl-LeX (sLeX), Lewis a (Lea), sialyl-Lea (sLea) and Lewis b (Leb) that share common topographies (Table 1), and fails to recognize LeY expressing tumor-derived cell lines [29]. These results suggest that LeY vaccines can lack sufficient specificity and may not direct the immune response against carbohydrate conformations expressed on neoplastic cells. Defining the configuration of native carbohydrate structures recognized by antibodies is important for understanding the basis of antigen specificity and is required to adequately assess the extent to which the same mechanisms for binding are used by peptide mimotopes [23].

The crystal structure of the anti-LeY antibody, BR96, solved in complex with a nonate methyl ester LeY tetrasaccharide [30], provide a molecular basis for LeY antigen recognition and specificity for some anti-LeY antibodies [26], and how this binding relates to peptide binding [24]. We had previously generated a model of LeY interacting with the monoclonal anti-LeY antibody BR55-2 using homology modeling techniques [26]. We wished to determine whether this model could be used to ascertain the extent to which peptide mimotopes of LeY can be structrally correlated with LeY binding for BR55-2. Our goal is to determine if cross-reactive peptides recognized by BR55-2 bind by the same mechanism as LeY; if so the basis of cross-reactivity would be structural. In this report, we describe the structural basis for the antigenic mimicry by some peptides for the LeY antigen. A structure-based computer screening approach was used to assemble an epitope map of potential amino acids that could interact with the BR55-2 combining site. The mapping confirmed the previously identified YRY and WRY motifs reactive with BR55-2, and also suggested a new putative seqeunce, FSLLW that adopted a β-turn structure within the BR55-2 combining site. The FSL residues spatially mimicked the Fucα1-3GlcNAc moiety of LeY in binding to BR55-2. The

accuracy of this approach was corroborated by the isolation of this putative sequence from a random peptide display library. Furthermore, vaccination with a peptide containing the FSLLW sequence induces a highly-specific anti-LeY response that distinguished the Fucα1-3GlcNAc from Fucα1-4GlcNAc linkage observed in histo-blood antigen homologoues. The results provide a molecular perspective that peptides can bind by similar mechanisms as the carbohydrate ligands that they mimic, inducing immune responses that are specific. These studies further demonstrate a rational approach for the identification of peptidomimetics, which can be used to direct the diversity of combinatorial libraries or optimize peptides that are selected for particular antibody subsets. This theoretical epitope mapping approach has general implications for studying protein interactions that share similar structural features, complementing combinatorial library screening approaches.

Results

Identification of BR55-2 binding site residues

Structural studies on Lewis antigens have generally substantiated that their conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes [26, 31-33]. These results further indicate that hard sphere or rigid-geometry calculations, albeit without solvent, provides a good picture of the steric repulsion that modulate the conformational properties of the Lewis antigens. Consequently, structure-based drug design approaches [34-36] offers the ability to establish potential interaction profiles (epitope map) to elucidate a molecular basis for peptide mimicry of the LeY antigen in binding to BR55-2.

To test this idea we performed a computer screening search with the program LUDI that resulted in 231 ligands identified to interact with the BR55-2 combining site. Table 2 summarizes the hydrogen bonding profiles of ligands for BR55-2, and identified contact residues within the BR55-2 combining site. The majority of contacts with LeY occur with main chain (MC) atoms, with some involvement with side chains (SC), as does interaction with the LUDI identified ligands. Of note, all BR55-2 residues that interact with LeY are identified in the LUDI search. Guanidinium type groups are observed to form bifucated hydrogen bonds with the same BR55-2 residue functional groups as the Fucα(1-3) moiety of LeY.

We have previously shown that LUDI could be used to map a LeY peptide mimic to the combining site of the anti-LeY monoclonal antibody B3 [24]. As a further illustration of this approach, definition of residue types shown in Table 2 can be combined with bulky hydrophobic amino acids occupying the LeY spatial volume. In figure 1a, representative non-overlapping organic ligands are shown positioned within the BR55-2 combining site relative to LeY. It is observed that Leu like groups can occupy the spatial volume of Fucα(1-3) and Gal moieties of LeY. A hydroxyl type residue is hydrogen bonded Asp 31 (MC) of the heavy chain, with a Trp like ligand hydrogen bonded with the side chain of Asn 52A of the heavy chain side. The Phe, Ser and Trp like ligands occupy the volume of the GalNAc moiety.

These relative positions can be used to form a putative peptide with the BR55-2 sequence FSLLW within the BR55-2 combining site as shown in Figure 1b in which the Ser and Trp hydrogen bonding schemes are retained. This binding mode conformation is similar to turn type conformations previously suggested for peptide binding to antibodies [3, 23-25] that serves to place the peptide sequence into a depression in the antibody combining site. Previous studies implicated the aromatic homologous putative sequence tracts WLY, WRY and YRY as mimics of the LeY antigen [4, 13]. Conformational studies indicate that the W/YR/LY and W/YPYmotifs can adopt beta turn type structures [3, 24, 25] suggesting a particular peptide structure is required for polysaccharide mimicry. The beta turn characteristics of mimicking peptides has been further implicated as a binding mode conformation as evidenced in a crystal structure of a peptide mimic of a cryptococcus carbohydrate epitope in complex with an anti-cryptococal antibody [23]. Root mean square deviation (RMS) of the BR55-2-beta turn peptide complex after minimization and dynamics calculations was found to be only .52 A compared with the BR55-2-LeY complex, indicating that the beta type II turn is readily accommodated within the BR55-2 combining site.

Reactivity of peptide mimics with BR55-2.

The LUDI results indicate that a variety of amino acid residues can certainly be found to interact with BR55-2, containing combinations of aromatic, bulky hydrophobic and hydroxyl containing residues. To further substantiate this conclusion, we screened a phage library containing a random 15 amino acid insert with BR55-2. Isolation of 100 random clones resulted in the identification of a variety of peptides containing these amino acid compositions. We identified 28 peptide families with selected family sequences

shown in Table 3. One peptide isolated displayed an exact match of the putative FSLLW sequence track (K61104 Table 3), while another contained a WRY sequence (K61109, Table 3).

We synthesized respective multiple antigen peptide (MAP) forms of the various peptides for detection of reactivity patterns with BR55-2. Evidence for Arg recognition by BR55-2 is demonstrated in ELISA assays in that substitution of Pro for Arg within the YRY sequence tract diminishes BR55-2 reactivity for the YPY containing peptide (Figure 2). The Arg containing peptides K61106 and K61107 (Table 3) bind to BR55-2 and compete with LeY for BR55-2 in a concentration dependent manner (Figure 2b), while the Pro substituted peptide K61105 (Table 3) displays diminished ELISA reactivity with BR55-2 (Figure 2a) and does not compete with LeY for BR55-2 binding (Figure 2b). These results indicate that a single substitution within a peptide sequence effects the antigenic mimicry of what are otherwise homologous peptides and that the presence of aromatic groups alone does not account for cross-reactivity.

The effect on binding with changing the presentation of a putative sequence tract is further observed with the peptide K61109 (Figure 2a) which contains the WRY sequence tract (Table 3). The general lack of reactivity of aromatic residue containing peptides is also observed with peptides K61108, K61110, K61111 and K61223 (Table 3). The peptides K61110 and K61111 were also isolated from a 15mer library with BR55-2. It was previously noted that it may be that library screening processes favor peptides that can assume conformations conducive to antibody binding when expressed on phage, but do not achieve the same conformation when synthesized free of the constraints imposed by the phage protein carrier [16, 37]. In contrast, the peptide K61104, also isolated with BR55-2, displays reactivity with BR55-2 in ELISA and can compete as effectively as the K61107 and K61106 peptides for LeY binding to BR55-2 (Figure 2).

The induction of anti-carbohydrate immune response.

Because the FSLLW sequence tract was identified both computationally and by phage display, we decided to test the immunological mimicry of the K61104 peptide, Balb/c mice were immunized with the K61104 MAP peptide administered with the adjuvant QS-21. The anti-K61104 sera was predominately of IgM isotype as observed in our previous studies using MAP peptides [38]. The anti-K61104 sera displayed a three to fold increase in reactivity for LeY over Leb, titering up to 1:2000 in ELISA (Figure 3a). At 1:50 serum dilution (Figure 3b), higher levels of reactivity are observed for LeY and LeY substituents with about the same level

of reduced reactivity for Leb hexasaccharide, LeX-pentasaccharide, sLeX, Lea, and sLea. Minimal binding is observed for a ubiquitous disaccharide unit Galβ1-3Gal.

To further define the minimal determinate that distinguishes selectively for LeY over its homologues, the serum was further screened against a variety of LeY constituents with the best reactivity observed with the Fucα1-3GlcNAc moiety (Figure 3b), reflecting the spatial association of the peptide for BR55-2 in comparison with other moieties on LeY (Fig 1b). Most importantly, the anti-K61104 sera distinguishes the Fucα1-3 from the Fucαa1-4 GlcNAc linkage, displaying significantly reduced reactivity with Fucα1-4GlcNAc. This selective interaction sets apart reactivities between Leb and LeY, since reactivity is observed for the H type 1 constituent of Leb (Figure 3b). The cross-reactivity of the anti-peptide serum for LeY in a specific manner suggests a structural mimicry between the K61104 peptide and LeY as indictaed in Figure 1b.

Discussion

Antigens, such as carbohydrates, that are not readily addressable by genetic vectors are a challenge in the design of effective vaccination approaches for many pathogens and perhaps in tumor immunity. Peptide mimotopes of carbohydrates provide an alternative vaccine approach. Peptide mimotopes have previously been used to better define the fine specificity of anti-carbohydrate antibodies [12, 16] and to elicit antibodies to protective carbohydrate epitopes [3, 4, 12]. The concept of using surrogate antigens as an immunogen requires that the antigenic mimicry, accomplished using amino acids in place of sugars, induces an immune specificity pattern for the nominal carbohydrate antigen that is specific [39-42].

The analysis of the finer details of mimicry between peptide and carbohydrate ligands is relevant for defining structural/function correlates necessary in vaccine design applications [23, 24, 43]. It is apparent that the structural rules governing molecular mimicry are required to be defined for the successful exploitation of peptide mimotopes. A more precise understanding of the binding of peptide mimotopes at the molecular level should provide insight as to whether the occurrence of motifs mimicking carbohydrate structures simply reflects an advantage provided by aromatic rings, for example, for interaction between proteins [16] or certain aromatic amino acid motifs can specifically mimic fifferent sugars.

As a model system, our initial focus has been directed towards peptides that are reactive with the anti-LeY MAb BR55-2 since the molecular recognition of BR55-2 for LeY is well described. Our goal is to determine if cross-reactive peptides recognized by BR55-2 bind by the same mechanism as LeY; if so the basis of cross-reactivity would be structural. Epitope mapping using LUDI indicates that both main chain and side chain atoms within the BR55-2 combining site function in antigen-antibody recognition (Table 2). Some of these potential contact sites are the same as those used by LeY. These contact sites were used to develop a model for peptide binding in which a putative peptide sequence, FSLLW, was de novo identified to adopt a β-turn within the BR55-2 combining site (Figure 1). The FSLLW sequence is positioned such that the FSL tract occupies the Fucα1-3GlcNAc position of LeY. In previous studies the identification of non-overlapping amino acid residue types could be used to position a peptide mimic of LeY within the anti-LeY B3 combining site in which the putative peptide sequence APWLY also adopted a type II beta turn [24]. Importantly, the FSLLW sequence was subsequently identified in a peptide isolated with BR55-2 from a peptide library (Table 3).

On the basis of three-dimensional analysis, the construction of an effective mimotope requires the improved fitting between bound peptide and the antibody to maximize particular interactions within the antibody heavy and light chains [23, 43]. The way in which the FSLLW sequence interacts with the BR55-2 heavy and light chain, emulating LeY binding, suggests this putative sequence can function as a LeY mimic (Figure 1). Screening of a peptide library with BR55-2 isolated clones having this sequence tract (Table 3). In contrast to other aromatic residue containing peptides isolated with BR55-2 (Table 3), the K61104 peptide competes with LeY for BR55-2 binding, implying that the peptide and carbohydrate binding sites overlap (Figure 2). Immunization with this peptide leads to a LeY specific immune response (Figure 3) that is mediated by selective interaction with the Fucα(1-3)GlcNAc moiety (Figure 3b).

Likewise, it appears that the monoclonal antibody BR55-2 can bind to the sequence tract W/YRY mediated by the Arg residue that mimics the spatial position of Fucα(1-3) by contacting the same atoms within BR55-2 (Table 2). The mimicry for Fucα(1-3) by the guanidinium group of Arg, as identified by the LUDI search, might be a basis for partial mimicry of LeY by W/YRY containing peptides (Figure 2). The single substitution of Pro for Arg in the W/YRY tract reduces BR55-2 binding (Figure 2), further

demonstrating that specificity of binding can be determined by the identity of the peptide side-chains that constitute the motif. BR55-2 did not bind to peptides containing WLY, WPY and WVF containing peptides (Table 3), further implying that aromatic residues alone are not sufficient for antibody binding. These results provide further evidence that peptides and carbohydrates can bind to the same antibody binding site, while changes in peptide presentation lend to fine specificities of anti-carbohydrate antibodies.

The structure of the complex of LeY with BR55-2 provides a molecular comparison for peptide binding in the antigen combining binding site and explains peptide competition for binding with LeY. Furthermore, these studies intimate that peptides can bind to isolating antibodies by the same mechanisms as the original carbohydrate antigen. This finding does not negate previous studies suggesting that peptides appear to be specific for their isolating monoclonal antibodies [12]. This conclusion was reached by comparing panels of antibodies that recognize the same carbohydrate epitope and consensus peptides isolated by the respective anti-carbohydrate antibodies. Peptide library screening with BR55-2 verifies the role played by W/YXY containing sequences in binding to BR55-2 (Table 3), but synthetic forms of peptides out-side the context of the tertiary constraints of the carrier protein may not cross-react with the isolating antibody (Figure 2). Furthermore, not all peptides isolated with antibodies have shown to be capable of induction of cross-reactive antibodies with the nominal antigen that the peptides are suppose to mimic [16, 19].

Systematic approaches involving computational as well as experimental tools have been used to analyze and exploit topological similarity between dissimilar structures [44-48]. New computer technologies may have an important impact on the discovery of ligands that bind to antibodies. Computer-based strategies exploit the structural information of the target molecules to propose novel therapeutic agents. In contrast to conventional approaches of random screening of large libraries, computational programs like LUDI provide an efficient automatic method to screen large databases of compounds to identify lead candidates and their corresponding three-dimensional positions within an antibody combining site. While many studies using this computerized screening strategy have been reported in the discovery of novel inhibitors [49], we have extended this approach to refine concepts associated with antigenic mimicry.

While current procedures for predicting ligand - antibody interactions are limited, mainly due to the conformational flexibility of ligands and antibodies and the role of solvent in mediating ligand recognition and binding, the utilization of a crystallographically determined starting position can, nevertheless, lend to discriminating differences in binding orientations of analogs. Three dimensional structures of antibodies in complex with peptides will facilitate structure-based design of peptide surrogates for vaccine applications. Strategies for improving the complementary between peptide mimotopes for antibody combining sites has been suggested [23]. The computer approach described here lends one more facet to the rational design process. Successful application of this area would be useful as an approach to further develop peptide mimics for Cryptococcus neoformans [23], Neisseria gonorrhoeae lipooligosaccharide epitopes in which no vaccine exists [50], improvement of our own peptide that mimics Neisseria meningitidis [3], and mimics for LeY as a cancer vaccine [38] and a perhaps an HIV vaccine [4]. The results presented here support the suggestion by Young et al that molecular imprinting can be sufficient to generate a molecular mimetisim from an immunological point of view [23].

Experimental Protocol

Epitope mapping of BR55-2 combining site

Using the positioned LeY structure in the BR55-2 combining site, we implemented the program Ligand-Design (LUDI [35] Biosym Technologies) to search a fragment library for amino acid residue types that can interact in a manner similar to LeY. A LUDI search was performed using standard default values and fragment library supplied with the program to identify fragment positions within the BR55-2 binding site. This program constructs possible new ligands for a given protein of known three-dimensional structure. Small fragments are identified in a database and are docked into the protein binding site in such a way that hydrogen bonds and ionic interactions can be formed with the protein and hydrophobic pockets are filled with lipophilic groups of the ligand. The positioning of the small fragments is based upon rules about energetically favorable non-bonded contact geometry's between functional groups of the protein and the ligand. The center of search was defined using the crystallographic LeY position. In this approach the OH-3^c position on the LeY structure was used as the sampling point. Peptides were built using InsightII (MSI/Biosym Technologies) and positioned relative to the docked LUDI fragments. The peptide backbone

and side chain torsional angles were rotated using a fixed docking algorithm within InsightII until the side chains of the peptide were approximate to the corresponding LUDI fragments. The peptide-BR55-2 complex was subjected to energy optimization and molecular dynamics simulations as with the LeY-BR55-2 complex as previously described [24].

Phage-display library screening

The pentadecamer library displayed on a filamentous phage (fd phage) surface protein (pIII) was obtained from Dr. George Smith. The 15mer library has been described [5, 8] and is constructed with the phage fUSE5 as the vector. Biopanning was performed as previously described [5, 8]. The phage particle epitope library was reacted with biotinylated BR55-2 immobilized on streptavidin-coated polystyrene petri dishes. In the first rounds, approximately 10^{12} TU (transducing units) were incubated overnight with 10 ug/ml bio-BR55-2 in 100 ul reaction mixtures. Phage eluted in the first rounds were amplified in liquid phase and subjected to a second round of affinity purification with BR55-2 at concentrations of 5ug/ml. Phage from the second round were amplified and subjected to further rounds with decreasing Ab concentration. The decrease in Ab concentration in the second and third rounds, and the use of excess phage introduce binding competition, and are intended to select for high-affinity epitopes [5, 8].

Reactivity with BR55-2 and control MAbs was measured by ELISA following established protocols [5, 8]. We have observed differences in antibody affinity for phage in coupling biotin to antibodies in ratios ranging from 1:2 to 1:22. In some cases coupling under high ratios severely reduced antigen (phage) binding. In our BR55-2 studies, we used a 1:2 molar ratio which reduced antigen detection in ELISA by only 10%. Percentage yield on a TU basis (percentage ratio of output phage to input phage) was determined for the eluted phage and of fd-tet after each round of biopanning. Fd-tet is used to determine background binding because its genome encodes wild-type pIII which does not encode the short peptide sequence that is displayed on the virion surface. Sequencing was performed using a rapid non-radioactive δTaq cycle PCR sequencing method [51].

Preparation of peptide antigens.

Peptides (Table 2) were synthesized as Multiple Antigen Peptides (MAPs) (Research Genetics, Huntsville Alabama) made by Fmoc synthesis on polylysine groups resulting in the presentation of 8 peptide clusters.

Preparation of Antibodies Against Carbohydrate-Mimicking Peptides

For generation of polyclonal sera, Balb/c mice (n=4 per group) 4-6 weeks of age, were immunized i.p. with 50 ug of the respective MAPs and 20ug of QS-21 adjuvant (Aquila Pharmaceuticals, Worcester MA), at intervals of 2 weeks for 6 weeks. Serum was collected at 7 and 14 days after the last immunization and stored at -20^{o}C.

ELISA assays

Solid phase ELISA was performed to assess the binding of anti-carbohydrate monoclonal antibodies and polyclonal anti-peptide sera to MAPs or a variety of carbohydrate synthetic probes incorporated into a polyacrylamide (PAA) matrix (Glycotech, Rockville Md.). For peptide ELISAs, MAPs were coated on Immulon 2 plates (2ug/well) and reacted with 0.2 ug of the anti-LeY monoclonal antibody BR55-2 developed against MCF-7 cells [52, 53]. For peptide inhibition, plates were coated overnight with LeY-PAA at 0.1 ug/well. The MAbs (0.1 ug) or serum was admixed with varying concentrations of MAPs for 15 min on ice, and then allowed to react with LeY coated plates. For serum evaluation of anti-carbohydrate activity, Immulon 2 plates were coated with a variety of carbohydrate probes that included Fucα1-4GlcNAc, LeY, Galβ1-3Gal, Galβ1-3GalNAc, sialyl-Lea, Lea, sialyl-LeX, LeX, LeX-pentasaccharide and Leb-hexasaccharide. Plates were coated with 2ug/well of the respective probes overnight at 4^{o}C and blocked [4]. Serial dilutions of the respective anti-sera was added and resolved with 100 μl / well of 1 : 10000 anti-Mouse isotype matched-HRP (Sigma) diluted in blocking buffer, incubated at 37^{o}C for 1hr. Absorbance at 450nm was read for all ELISAs using a Dynatech MR5000 ELISA reader after 15 min of color development. All results were calculated from triplicate measurements.

Acknowledgment

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Figure Legends

Figure 1. Identification and placement of the putative FSLLW sequence interacting with BR55-2. In figure 1a space filling of BR55-2 (gold color) is shown with non-overlapping residue types identified by LUDI in comparison with LeY within the BR55-2 combining site. In figure 1b illustrates optimized FSLLW peptide in the BR55-2 combining site relative to the LUDI identified fragments.

Figure 2. Reactivity of putative motifs by ELISA. (A) Reaction of BR55-2 with respective MAP peptides. (B) Inhibition of MAb BR55-2 binding to solid-phase LeY-PAA by soluble MAP peptides. A constant amount of BR55-2 was incubated with increasing amounts of MAP peptides, and then reaction of free MAb with LeY was measured by ELISA. Data points reflect 50% inhibition at 2.3 uM of K61104 peptide, 1.6 uM for K61106 and K61107 pepide inhibitors as measured by reduction of Absorbance value in ELISA.

Figure 3. Reactivity of the K61104 serum with neolactoseries constituents. A.) Titration of the IgM portion of the derived anti-peptide sera with LeY and Leb. B.) Profile of cross-reactivity of anti-peptide MAP sera with Lewis carbohydrate probes.

Table 1 Neolactoseries core antigen structures

Antigen	Structure
LeY	(Fucα1-2)Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Leb	(Fucα1-2)Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Type 1 chains	(Fucα1-2)Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Type 2 chains	(Fucα1-2)Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer
LeX	Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
sLeX	NeuNAca2-3Galβ1→4(Fucα1-3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Lea	Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer
sLea	NeuNAca2-3Galβ1→3(Fucα1-4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer

Table 2 Ligand/Epitope Hydrogen Bond Contact Residues on BR55-2

	BR55-2	
Amino Acid Types Identified Computationally	***Light Chain***	***Heavy Chain***
Guanidinium		Ala100(MC), Met96(MC), Tyr35(SC) Asn52A(SC), Asp31(MC) Ala 100(MC), Met96(MC) Asp97(MC), Met96(MC)
Tyr	Ser92(MC),	Tyr35(SC), Asp31(SC), Asp31(MC) Asp97(MC), Ser92(MC)
Trp		Ala100(MC), Met96(MC), Tyr33(SC) Asp31(MC), Asn 52a
Ser/Thr	Ser27E(SC)	Asp31(MC), Asp97(MC), Tyr50(SC)
His		Tyr32(SC), Tyr35(SC), ASP97(MC) Asp31(MC), Tyr50(SC)
NH/CO	His27D, Tyr32(SC)	Tyr35(SC), Asp31(MC), Gly53(MC) Tyr32(SC), Asn52a(SC)
LeY tetrasaccharide Identified Crystallographically		
Gal	His27D	Tyr35(SC)
GlcNAc		Tyr33(SC)
Fucα (1-2)	Ser27E	
Fucα(1-3)		Ala100(MC), Met96(MC), Tyr35(SC)

Table 3 Peptides Described in These Studies

Designation	Sequence Motif	Structure	Method of Identification	BR55-2 reactivity	LeY Inhibition
K61105	YPY	GGIYYPYDIYYPYDIYYPYD	Synthetic Design	+	-
K61106	WRY	GGIYWRYDIYWRYDIYWRYD	Synthetic Design	++	++
K61107	YRY	GGIYYRYDIYYRYDIYYRYD	Synthetic Design	++	++
K61108	WLY	GGGAPWLYGGAPWLYAPWLY	Synthetic Design	-	-
K61223	WLY	GGGAPWLYGAPWLYGAPWLY	Synthetic Design	-	-
K61109	WRY	GGARVSFWRYSSFAPTY	Phage	-	-
K61110	WPY	GGGWPYLRFSPWVSPLG	Phage	-	-
K61111	WVF	GGAGRWVFSAPGVRSIL	Phage	-	-
K61104	FSLLW	IMILLIFSLLWFGGA	Phage	+	++

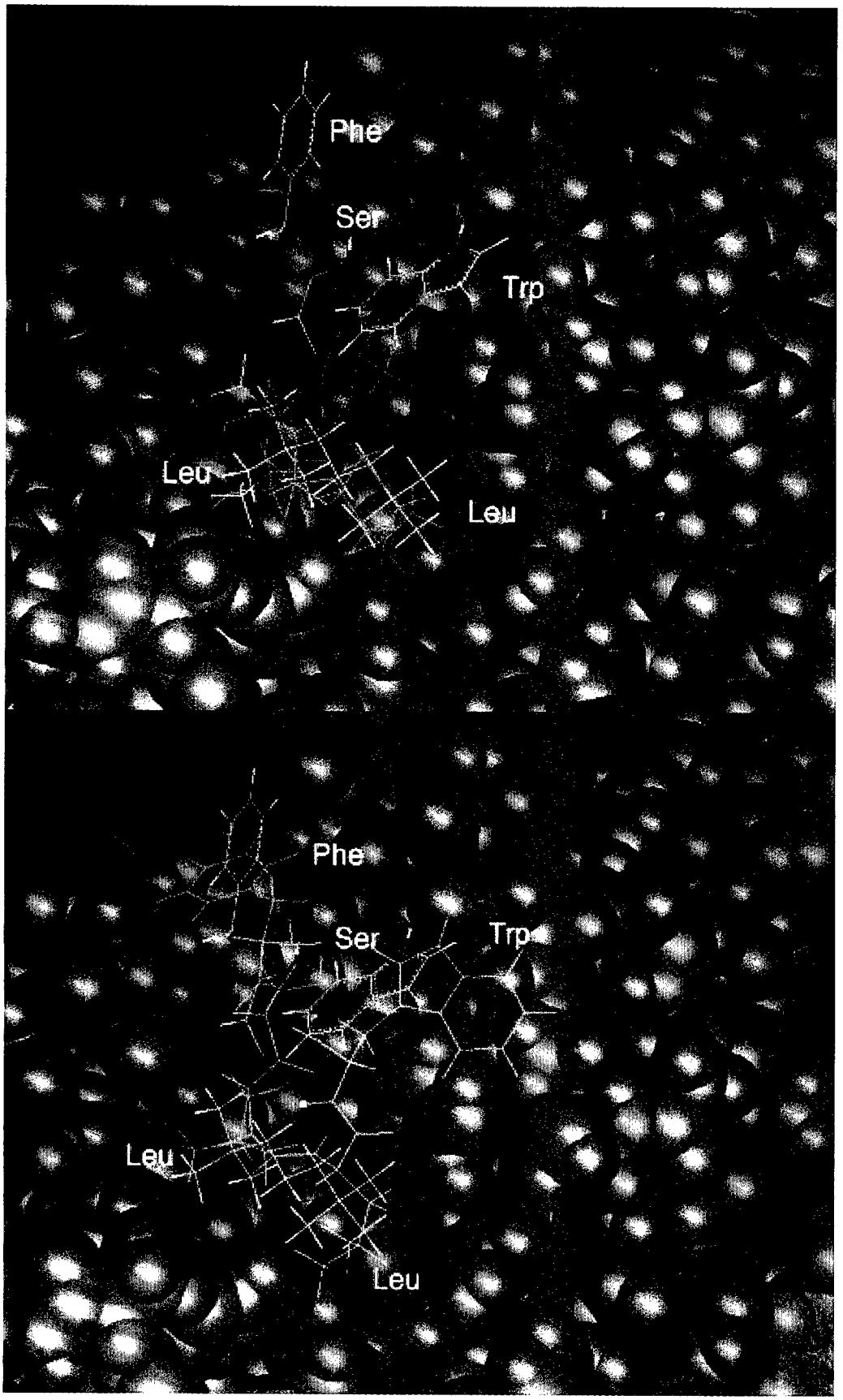

Figure 1

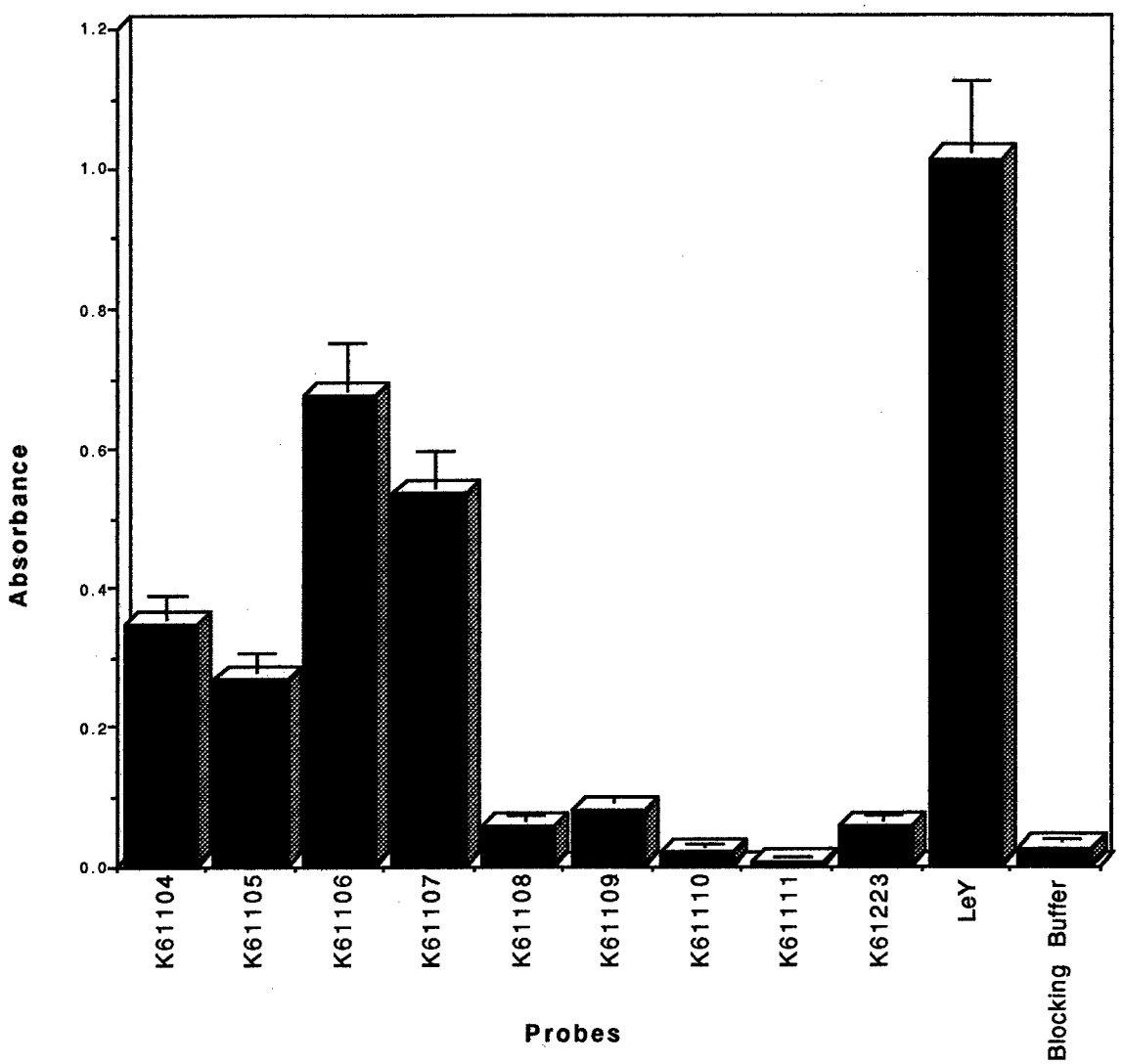
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
K61104
K61105
K61106
K61107
K61108
K61109
K61110
K61111
K61223
LeY
Blocking Buffer
Probes

Figure 2a

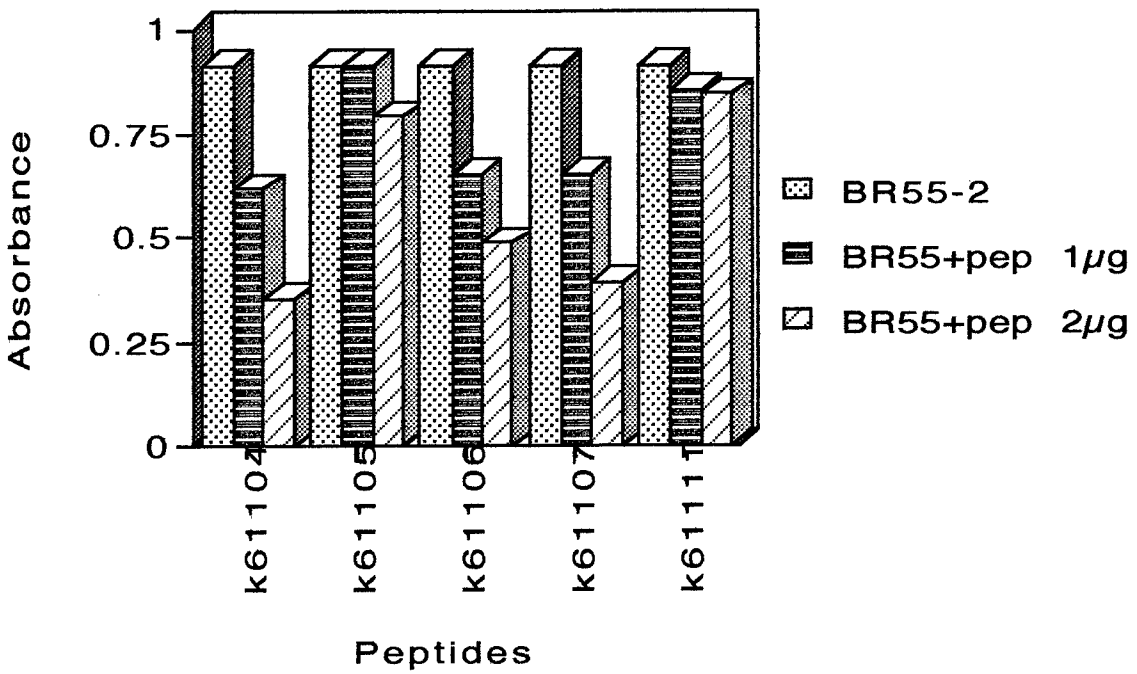
Absorbance
1
0.75
0.5
0.25
0
k61104
k61105
k61106
k61107
k61111
Peptides
BR55-2
BR55+pep 1μg
BR55+pep 2μg

Figure 2b

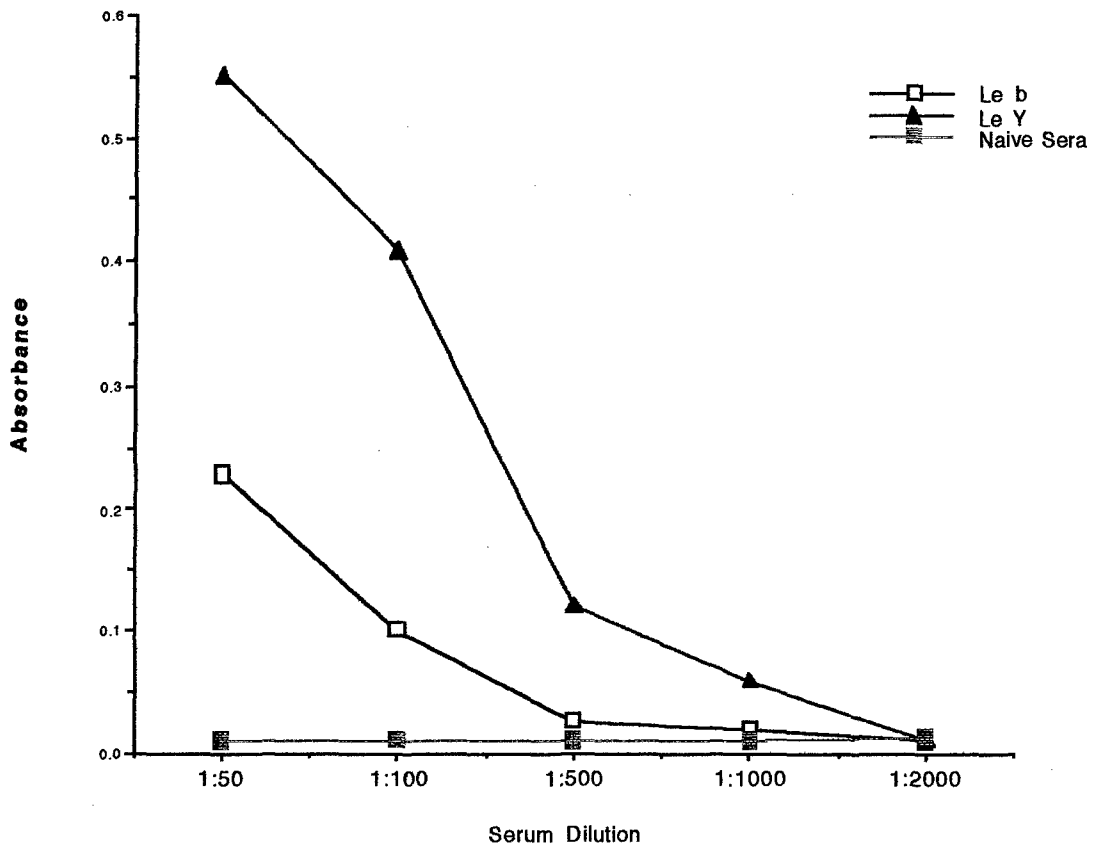
Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1:50
1:100
1:500
1:1000
1:2000
Serum Dilution
Le b
Le Y
Naive Sera

Figure 3a

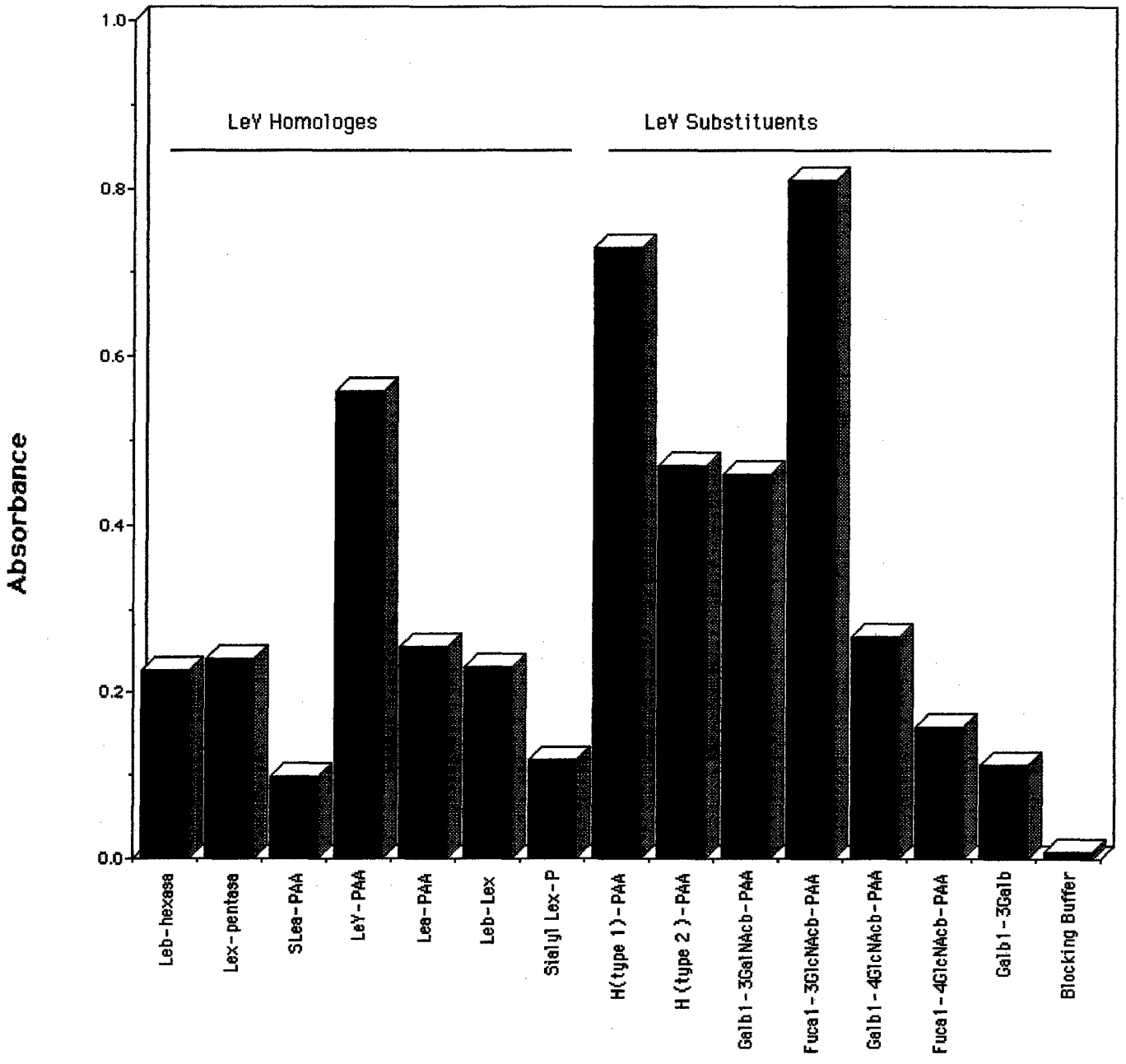
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
LeY Homologes
LeY Substituents
Leb-hexasa
Lex-pentasa
SLea-PAA
LeY-PAA
Lea-PAA
Leb-Lex
Sialyl Lex-P
H(type 1)-PAA
H (type 2)-PAA
Galb1-3GalNAcb-PAA
Fuca1-3GlcNAcb-PAA
Galb1-4GlcNAcb-PAA
Fuca1-4GlcNAcb-PAA
Galb1-3Galb
Blocking Buffer

Figure 3b.

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Molecular Immunology

Antigenic and immunological mimicry of peptide mimotopes of Lewis carbohydrate antigens

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Abstract

Peptides may substitute for carbohydrates in reactions with carbohydrate-specific molecules. Recently, we found that peptides containing aromatic residues mimic mucin and histo-blood group related carbohydrate epitopes, eliciting polyclonal responses cross-reactive with bacterial and viral antigens that express these carbohydrate forms. These results demonstrate that peptides can function in in vivo and in vitro models as carbohydrate surrogate antigens. To further explore the nature of the antigenic and immunogenic properties of such mimotopes, synthetic peptides with aromatic amino acids were tested to delineate reactivity patterns with several anti-neolactoseries monoclonal antibodies (MAbs). These MAbs recognize biologically important conformations of the histo-blood group related Lewis antigens expressed on the surface of a variety of human cancers. Results by ELISA demonstrate that the MAbs can distinguish particular peptide motifs that include the sequences GGIYYPYDIYYPYDIYYPYD, GGIYWRYDIYWRYDIYWRYD and GGIYYRYDIYYRYDIYYRYD. Substitution of Arg by Pro diminished the reactivity of the anti-Lewis Y (LeY) MAb BR55-2. Binding of LeY to BR55-2 was inhibitable by the Arg containing peptides. Serum against all three peptides displayed reactivity with synthetic histo-blood group related antigen probes. Immunologic presentation of the peptides as multiple antigen peptides (MAPs) improved peptide ability to induce LeY specific immune responses. Serum bound to human tumor cells that preferentially expressed neolactoseries antigens, but not to normal tissues. Immunoprecipitation of human breast tumor cell lysates before and after treatment with tunicamycin confirmed serum carbohydrate binding. The anti-peptide sera mediated tumor cell killing by complement mediated cytotoxicity. These results indicate that mapping peptide epitopes with anti-carbohydrate antibodies can lend to defining antibody fine specificities that can go undetected by screening of carbohydrate antigens alone. In addition, these results confirm that peptides and carbohydrates can bind to the same antibody binding site and that peptides can structurally mimic salient features of carbohydrate epitopes.

Keywords: Peptide mimotopes; Carbohydrate antigens; Lewis Y; Vaccine; Cancer

1. Introduction

The production of specific antibodies against polysaccharides play a pivotal role in the defense against gram-negative and gram-positive bacteria, and have shown promise in vaccine approaches in the immunotherapy of cancer (Livingston et al., 1997; Livingston and Ragupathi, 1997; Ravindranath et al., 1997aRavindranath et al., 1997b). However, carbohydrate antigens are typically poorly immunogenic, very difficult to purify in large quantities, difficult to synthesize and usually induce mostly short-lived IgM type antibodies in a vaccinated host without long lasting immunity. Most carbohydrate antigens belong to the category of T cell-independent antigens that reflect their inability to stimulate MHC class II-dependent T cell help (Mond et al., 1995). Consequently, carbohydrates alone are not capable of induction of a sufficient anamnestic or secondary immune response and require extensive adjuvanticity. An alternative approach for augmentation of carbohydrate immunity is the use of peptide or polypeptide surrogate antigens.

The idea of using surrogate antigens as immunogens, requires that antigenic mimicry, accomplished using amino acids in place of sugars, induces a precisely reproduced immune specificity pattern for the nominal antigen (Olsson, 1987; Diakun and Matta, 1989; Hutchins et

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al., 1996; Tsuyuoka et al., 1996). Mimicking peptides represent a new and very promising tool to overcome T cell-independence and to increase the efficiency of the immune response to carbohydrates (Shikhman and Cunningham, 1997). Peptides with a high prevalence of tryptophan and tyrosine occur in peptides that mimic carbohydrates (Oldenburg et al., 1992; Scott et al., 1992; Hoess et al., 1993; Shikhman et al., 1994; Shikhman and Cunningham, 1994; Westerink et al., 1995; Valadon et al., 1996; Harris et al., 1997; Zhang et al., 1997b; Agadjanyan et al., 1997; Phalipon et al., 1997; Taki et al., 1997). A more precise understanding of the binding properties of carbohydrate-mimicking peptides is required to determine whether the occurrence of aromatic containing motifs is due to molecular mimicry or simply reflects an advantage provided by aromatic rings for interaction between proteins (Valadon et al., 1996).

Peptides with the aromatic motif W/YXY have been previously defined to mimic several carbohydrate subunits that include YPY as a mimic of mannose as identified from peptide phage screening with Con A (Oldenburg et al., 1992; Scott et al., 1992) WRY found to mimic α(1–4)glucose as identified from analysis of protein that bind to α-amylase (Murai et al., 1985; Mirkov et al., 1995), WLY found to mimic Lewis Y (LeY) as identified from peptide phage screening with an anti-LeY antibody, B3 (Hoess et al., 1993) and YRY derived from an anti-idiotypic antibody found to mimic the major C polysaccharide α(2–9) sialic acid (MCP) of *Neisseria meningitidis* (Westerink et al., 1995). This aromatic motif is also implicated as a mimic for a *Cryptococcus neoformans* epitope (Valadon et al., 1996).

The sequence similarities that define this motif suggest that antibodies to homologous peptides might cross-react with similar subunits expressed on what are otherwise dissimilar carbohydrate structures; if so the basis of cross-reactivity would be structural. Mapping peptide epitopes containing the W/YXY motif with anti-carbohydrate antibodies could lend to defining fine specificities that might go undetected by screening carbohydrate antigens alone. Molecular modeling suggests that the neolactoseries LeY and sialyl-lex (sLeX) tetrasaccharide structure is similar to the core structure of MCP, providing a structural basis for potential cross-reactivity of antibodies (Agadjanyan et al., 1997). Consequently, evaluating responses to neolactoseries antigens would further define recognition specificities for antibodies directed to these important carbohydrate forms.

Here, we delineate specificity patterns associated with the antigenic and immunological mimicry of peptide mimotopes that induce antibodies to Lewis (Le) antigens. The blood group-related neolactoseries carbohydrate structures Lewis X (LeX), sLeX, Lewis a (Lea), sialyl-Lea (sLea) and LeY are examples of terminal carbohydrate structures related to tumor prognosis (Miyake et al., 1992; Dabelsteen, 1996). These antigens (Figure 1) constitute carbohydrate moieties on some tumor associated gangliosides, the human carcinoembryonic antigen family, the human pancreatic MUC-1 antigen and identified on glycoproteins and glycolipids on carcinomas of the skin, stomach, pancreas, lung, colon, breast and prostate. SLeX and sLea, are implicated as immunogenic antigens in human melanoma as well (Ravindranath et al., 1997a). Histo-blood group related antigens are observed only at the secretory borders on normal tissues (Zhang et al., 1997a). This location appears to be inaccessible to the immune system, inducing neither tolerance nor autoimmune responses. Consequently, some Le antigens, notably LeY, are excellent targets for passive immunotherapy or a vaccine approach in the treatment of cancer.

Contrasting the antigenic and immunological properties of peptide mimotopes forms provides information about how amino acid differences lend to specific carbohydrate mimicry. We observe that the immunological presentation of W/YXY containing peptides influences the specificity pattern for histo-blood group synthetic carbohydrate probes. We also observe that the peptide mimotopes elicit polyclonal sera that specifically bind to human tumor cells, but not to normal tissues, and can mediate complement dependent cytotoxicity (CDC) of representative human breast cell lines, albeit at low titers.

2. Materials and methods

2.1. Preparation of peptide immunogens

Several peptides were synthesized, repeating putative centralized motifs, suggested to mimic carbohydrate forms and correspond to GGIYYPYDIYYPYDIYYPYD (K61105), GGIYWRYDIYWRYDIYWRYD (K61106), GGIYYRYDIYYRYDIYYRYD (K61107), GGGAPWLYGAPWLYGAPWLY (K61223), GGAPWLYGGAPWLYAPWLY (K61108). Other peptides were synthesized as variants that include GGAGRWVFSAPGVRSIL (K6111), GGGWPYLRFSPWVSPLG (K61110), GGARVSFWRYSSFAPTY (K61109). Peptides were synthesized with the addition of a tripeptide YGG spacer and a cysteine at the amino terminus conjugated to a lauroyl group (Westerink et al., 1995) (Bio-Synthesis, Lewisville, TX) or as multiple antigen peptides (MAPs) (Research Genetics, Huntsville, AL). For the proteosome conjugates, the meningococcal outer membrane proteins (or proteosomes) were prepared and complexed to the Lauroyl–C-YGG-Peptides as described by Lowell et al. (1988a, 1988b) in a 1:1 ratio, combining the components in the presence of detergent. The detergent was removed by extensive dialysis (Lowell et al., 1988a, 1988b). The lauroyl group allows for hydrophobic complexing of the peptide to the proteosomes

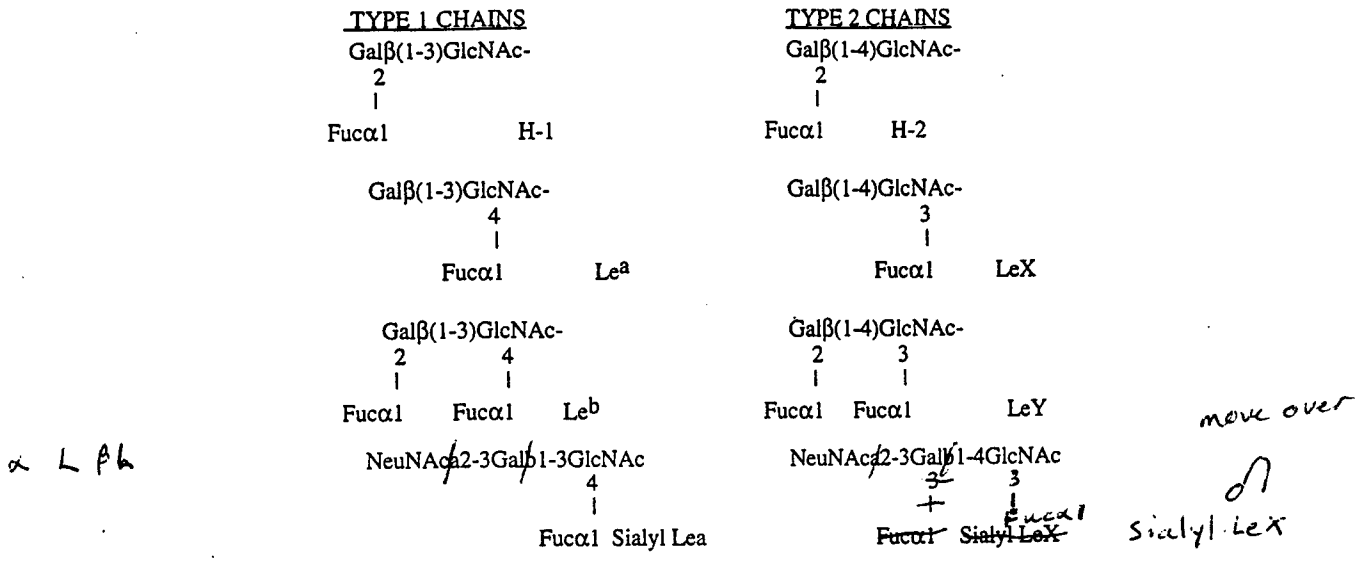

Fig. 1. Structures of H, Lea, Leb, LeX and LeY blood group determinants. Fuc: L-fucose; Gal: D-galactose; GlcNAc: *N*-acetylglucosamine, NeuNAc: sialyl derivative.

while the cysteine at the N terminus appears essential for immunogenicity apparently cross-linking multiple epitopes. Multiple antigen peptides (MAP) were made by Fmoc synthesis on polylysine groups, resulting in the presentation of eight peptide clusters (McLean et al., 1992; Rajadhyaksha et al., 1995).

2.2. Preparation of antibodies against carbohydrate-mimicking peptides

For generation of polyclonal sera, Balb/c mice ($n=4$ per group) 4–6 weeks of age, were immunized i.p. on a weekly basis for three weeks with 50 μg of the peptide–proteosome complex as described (Westerink et al., 1995). Other groups of mice were administered 50 μg of the respective MAPs and 20 μg of QS-21 adjuvant (Aquila Pharmaceuticals, Worcester MA), at intervals of two weeks for six weeks. Serum was collected at 7 and 14 days after the last immunization and stored at −20°C.

2.3. ELISA assays

Solid phase ELISA was performed to assess the binding of anti-carbohydrate monoclonal antibodies and polyclonal anti-peptide sera to MAPs or a variety of carbohydrate synthetic probes incorporated into a polyacrylamide (PAA) matrix (Glycotech, Rockville, MD). For peptide ELISAs, MAPs were coated on Immulon 2 plates (2 μg/well) and reacted with 0.2 μg of the anti-LeY monoclonal antibodies BR55-2 and 15.6, developed against MCF-7 cells (Steplewski et al., 1990; Scholz et al., 1991) or the anti-GD2/GD3 antibody ME361 (Iliopoulos et al., 1989). For peptide inhibition, plates were coated overnight with LeY–PAA at 0.1 μg/well. The MAbs (0.1 μg) or serum was admixed with varying concentrations of MAPs for 15 min on ice and then allowed to react with the LeY-coated plates. For serum evaluation of anti-carbohydrate activity, Immulon 2 plates were coated with a variety of carbohydrate probes that included Fucα1–4GlcNAc, LeY, Galβ1–3Gal, Galβ1–3GalNAc, sialyl–Lea, Lea, sialyl–LeX, LeX, LeX–pentasaccharide and Leb–hexasaccharide. Plates were coated with 2 μg/well of the respective probes overnight at 4°C and blocked (Agadjanyan et al., 1997). Serial dilutions of the respective anti-sera were added and resolved with 100 μl/well of 1:10,000 anti-mouse isotype matched-HRP (Sigma) diluted in blocking buffer, incubated at 37°C for 1 h. Absorbance at 450 nm was read for all ELISAs using a Dynatech MR5000 ELISA reader after 15 min of color development. All results were calculated from triplicate measurements.

2.4. Flow cytometry

Representative human LeY expressing cell lines include the breast cancer lines SKBR3, SKBR5, MCF7 and OVAR-3 obtained from ATCC (Rockville, MD). Control cell lines include the human non-LeY expressing cell line HS578 Bst (normal breast cell line, ATCC) and the human melanoma line WM793 (gift from D. Herlyn, Wistar Institute), SKMEL-28 (ATCC) and NIH3T3 murine fibroblast. For the preparation of cells, 10 ml of FACS buffer was added and the cells were washed, scrapped and transferred to 15 ml centrifuge tubes. Viable

cells were counted using trypan blue as indicator. Cells were diluted to 2×10^6/ml and 100 μl used for each sample. Primary sera (10 μl) were added to the sample tubes and incubated on ice for 30 min, washed twice with 1 ml FACS buffer and centrifuged for 5 min at 1500 rpm. 10 μl of FITC Ab (goat anti-mouse IgG or IgM FITC labeled (Sigma) diluted 1:20 with PBS) was added to the sample and incubated on ice for 30 min and again washed twice with FACS buffer. Cells were fixed using 2% paraformaldelhyde, followed by FACS measurement on a Becton Dickinson flow cytometer FACScan (Becton Dickinson, Los Angeles). Cells were also treated with neuraminidase to remove sialic acid residues on carbohydrates expressed on the cell surface. In these assays 4×10^7 cells/ml were treated with neuramindase (0.1 unit/ml in RPMI 1640) on ice for 2 h. Cells were spun down and ice cold medium, containing 0.1 mg/ml Fetuin, was added and incubated on ice for 15 min. Cells were washed with cold medium and reacted with antibody as described above.

2.5. Identification of tumor cell surface expressed LeY

Neoglycoproteins expressing LeY were detected by immunoprecipitation. SKBR3 cells were plated at 3×10^6/flask (T-75 flask) 24 hours before labeling. Medium was removed and replaced with 4 ml of methionine and cysteine-free Dulbecco's modified Eagle's medium containing 10% fetal bovin serum and incubated at 37°C for 60 min. Cells were then labeled biosynthetically with 200 μCi of ^{35}S methionine and ^{35}S cysteine for 4 h. To one group of cells, 5 ml of complete media was added and incubated for 2 h at 37°C with the glycosylation inhibitor tunicamycin (10 μg/ml). Labeled cells were harvested and washed with PBS. Immunoprecipitation was performed as previously described (Silver et al., 1987) with some modification. Cells were extracted with 0.02 mol/L Tris–HCL buffer, pH 7.2, containing 1% Triton X-100 (Sigma, St. Louis, MO) for 60 min at 4°C and extracts were cleared of particulate debris by sedimentation at 10,000*g*. Five hundred microliter aliquots of the supernatants were incubated with 4 μl of normal mouse serum for 15 min at 4°C, followed by an incubation with 100 μl of a 10% suspension of fixed *Staphylococci* (Pansorbin; Calbiochem, San Diego) for 30 min at 4°C. After sedimentation at 12,000*g* to remove the *Staphylococci*, 15 μg of antibody or 6 μl of sera were added to the cleared supernatant and the incubations were continued at 4°C for 1 h. Immune complexes were collected by adding 50 μl protein A and 50 μl protein G beads. After centrifugation the pellet was washed three times with DOC–10 mM NaCl and DOC–300 mM NaCl. The washed pellets were resuspended in 100 μl SDS-PAGE sample buffer (10 mM tris and 3% SDS pH 6.8 with 60 mg/ml DTT) boiled for 4 min and centrifuged. The supernatant was run on an 8% SDS-polyacrylamide gel.

2.6. Distribution of carbohydrate reactivities on human tissues

To further our studies of cross-reactivities, we have initiated screening our sera with human surgical specimens. We performed immunostaining (using an indirect immuno-peroxidase method) of tissue specimens derived from a variety of tumor types and normal tissue following procedures previously described (Garin et al., 1989). Tissues were obtained from the tissue procurement section of the Hospital of the University of Pennsylvania. Rabbit polyclonal anti-sera, raised against the K61107 peptide-proteosome conjugate, were used in this study. Serum was collected after the third immunization. This serum proved to be positive for LeY. As a control antibody we used MAb BR55-2. Control immunocytochemical experiments were performed by substitution of primary rabbit serum with normal rabbit serum at the same dilution. Formalin-fixed, paraffin-embedded normal tissue sections, representing the major organ systems, were selected from surgical diagnostic files. Fresh normal and tumor human tissues (i.e. tonsil, skin, colon, skeletal muscle and normal tissue surrounding excised tumors) obtained at surgical resection and snap frozen in liquid nitrogen-cooled isopentane, were also examined. Immunocytochemistry was performed following procedures previously described (Garin et al., 1989).

Fresh tissues were embedded in OCT (Miles) before storage at −70C. Serial sections from frozen and wax-embedded tissues cut at 5 and 4, respectively are mounted on poly (L-lysine) coated slides, air dried and fixed in acetone (4°C, 10 min) before use in immunohistochemistry. Immunostaining was performed using a sensitive three-layer avidin–biotin complex (ABC) method with the rabbit IgG Vectastain Elite ABC (peroxidase) kit, as outlined by the manufacturer (Vector Laboratories). Control immunocytochemical experiments were performed by substitution of primary rabbit sera, with normal rabbit serum at the same dilution. Before application of normal goat serum, fixed and unfixed frozen sections were immersed in PBS, pH 7.4. Similarly, paraffin sections are dewaxed in xylene and hydrated through graded alcohols to water, then subsequently placed in PBS. Primary anti-sera were diluted to 1:100. After overnight incubation at 4°C, sections are rinsed in PBS and incubated for 1 h at 22°C with biotinylated goat anti-rabbit antibody. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 min; sections were rinsed in PBS and incubated for 30 min with ABC solution. After further washes in PBS, the reaction product was visualized using diaminobenzidine (Sigma) as chromogen. Sections were counterstained with Harris' hematoxylin, dehydrated with graded alcohol's, cleared in xylene and mounted.

Table 1
Peptide motifs that mimic carbohydrate structures

Motif	Carbohydrate	Structure	Reference
YYPY	mannose	methyl-α-D-mannopyranoside	(Oldenburg et al., 1992; Scott et al., 1992)
WRY	glucose	α(1–4)glucose	(Murai et al., 1985; Mirkov et al., 1995)
PWLY	Lewis Y	Fucα1→2Galβ1→4(Fucα1→3)GlcNAc	(Hoess et al., 1993)
YYRY	group C polysaccharide	α(2–9)sialic acid	(Westerink et al., 1995)

2.7. Complement-dependent cell cytotoxicity (CDC)

Sera were tested for their ability to bind to tumor lines and modulate CDC as previously described (Kitamura et al., 1994). Briefly, 10 μl of each cell line (4×10^4 cells per ml) were added to duplicate wells of microtiter plates and incubated overnight at 37°C. Medium was removed and 20 μl of serially diluted sera was added and incubated for 45 min on ice. Twenty μl of rabbit complement (1:2) was added. After 4 h, plates were fixed with methanol for 10 min, rinsed in distilled water, stained with 2% Giemsa stain in phosphate-buffered saline for 25 min and rinsed. Plates were counted under a light microscope and the percentage of cytotoxicity (PC) of a given serum dilution was calculated as follows: percentage cytoxicity = [1 − (number of cells in well treated with serum and complement/number of cells in well treated with medium only)] × 100. Control wells did not contain antisera.

3. Results

3.1. Antigenic mimicry of peptide motifs

To determine the extent of antigenic mimicry of the W/YXY putative motifs in shown in Table 1, a variety of peptides were synthesized, some repeating the respective putative centralized motifs. MAP forms were synthesized for detection of reactivity patterns with the anti-LeY monoclonal antibodies BR55-2 and 15.6A and the anti-sialylated LeX reactive antibody FH-6 (Figure 2a). These antibodies are very specific for their respective antigens. It is hypothesized that MAP forms may represent clustered configurations of the carbohydrate epitopes. In ELISA assays, significant BR55-2 and FH-6 reactive sequences were observed for the peptides, GGIYWRYDIYWRYDIYWRYD (K61106) and GGIYYRYDIYYRYDIYYRYD (K61107).

The observed reactivity profiles suggest that K61106 and K61107 mimic salient features of the surface conformation of LeY and Sialyl–LeX, that is compatible with the BR55-2 and FH-6 combining site, since these two antibodies selectively cross-react with these peptides. The substitution of Trp for Tyr appears not to significantly alter the reactivity of BR55-2 and FH-6 for K61106 and K61107, but substitution of Pro for Arg reduces their reactivity and abolishes binding to 15.6A. The reduction in reactivity of 15.6A for peptides otherwise reactive with BR55-2, suggests that the peptides mimic a structural feature(s) unique to BR55-2 recognition. BR55-2 and 15.6A display distinct binding properties for LeY expressing tumor cells. In addition, BR55-2 displays little reactivity with the K61223 and K61108 peptides that represent the APWLY motif reactive with the anti-LeY antibody B3 (Hoess et al., 1993). The effect of sequence on antibody recognition is observed with K61109, in which the WRY tract was synthesized in a different molecular environment. Inhibition of LeY–PAA binding of BR55-2 by these MAP peptides is shown in Fig. 2b. K61106 and K1107 displayed 50% inhibition of BR55-2 binding to LeY with 20 times molar excess. These data indicate that the YRY and WRY motifs, synthesized as a triplet, lend to their reactivity as LeY mimics in binding to BR55-2 that is mediated by a single amino acid substitution.

3.2. The induction of anti-carbohydrate immune responses

The immunological presentation of the putative motifs (i.e. short or longer peptides, presentation in a helix or beta bend) might mimic overlapping epitopes on otherwise different carbohydrate structures. To test this idea, Balb/c mice were immunized with peptide-proteosome conjugates representative of the motifs YPY (P1) and YRY (P2) or the same peptides as MAP forms administered with the adjuvant QS-21. Serum was collected one week after the last immunization, pooled and tested for LeY and Leb reactivity by ELISA. For the proteosome conjugates, we found that serum developed from the immunizations react with the two multivalent probes, with the IgG reactivity titering up to 1:2000 (Figure 3). Superposition of LeY and Leb structures indicate that despite the change of glycosidic linkage from β1–3 to β1–4 in the type 1 and 2 chains Fig. 1 they share a common topography (Thurin-Blaszczyk et al., 1996). The only effective difference is the position of the *N*-acetyl and hydroxymethyl groups projected on opposite sides of the type 1 and 2 difucosylated structures Fig. 1. The ELISA results in Fig. 3 suggest that the sera are reacting with this common topography. As expected the anti-sera

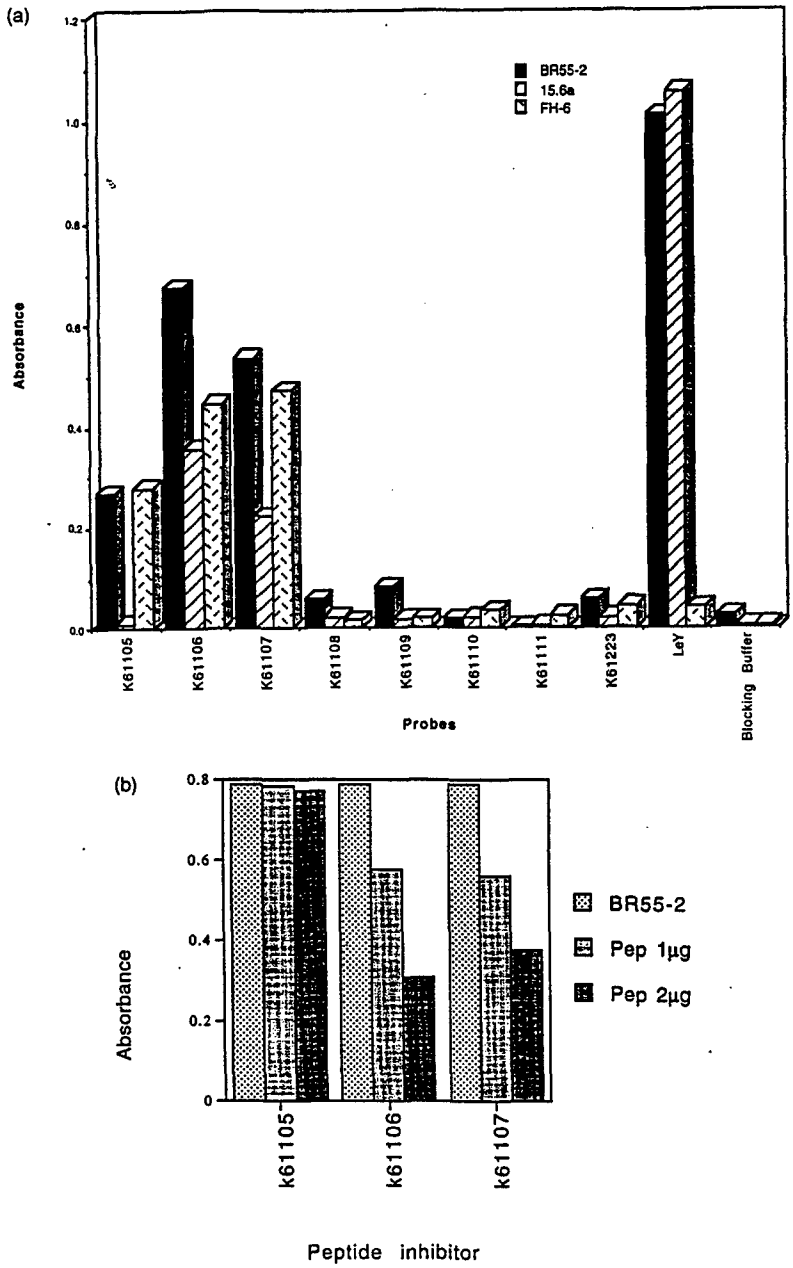

Fig. 2. Reactivity of putative motifs by ELISA. (a) Reaction of MAbs with respective MAP peptide forms. (b) Inhibition of MAb BR55-2 binding to solid-phase LeY–PAA by soluble MAP peptides. Constant amount of BR55-2 were incubated with increasing amounts of MAP peptides and then reaction of free MAb with LeY was measured by ELISA. Data points reflect 50% inhibition at 2 µg/ml of peptide inhibitor as measured by reduction of absorbance value in ELISA.

reacted to a similar extent with all of the Lewis structures tested (data not shown).

In contrast to the proteosome-peptide conjugates, increased specificity for LeY is observed for the MAP forms (Figure 4). At 1:50 serum dilution, the LeY reactivity of the anti-peptide sera is approximately 2–3 fold more reactive with LeY than with Leb-hexasaccharide, Lex-pentasaccharide, sLea and sLex. Considering the diminished reactivity against LeX that shares the Galβ1→4(Fucα1→3)GlcNAcβ1 component Fig. 1 it would appear that a fraction of antibodies reacts with a conformational component of LeY, providing for its increased reactivity, or alternatively, the affinity with LeY is increased. For either reason, these results suggest that

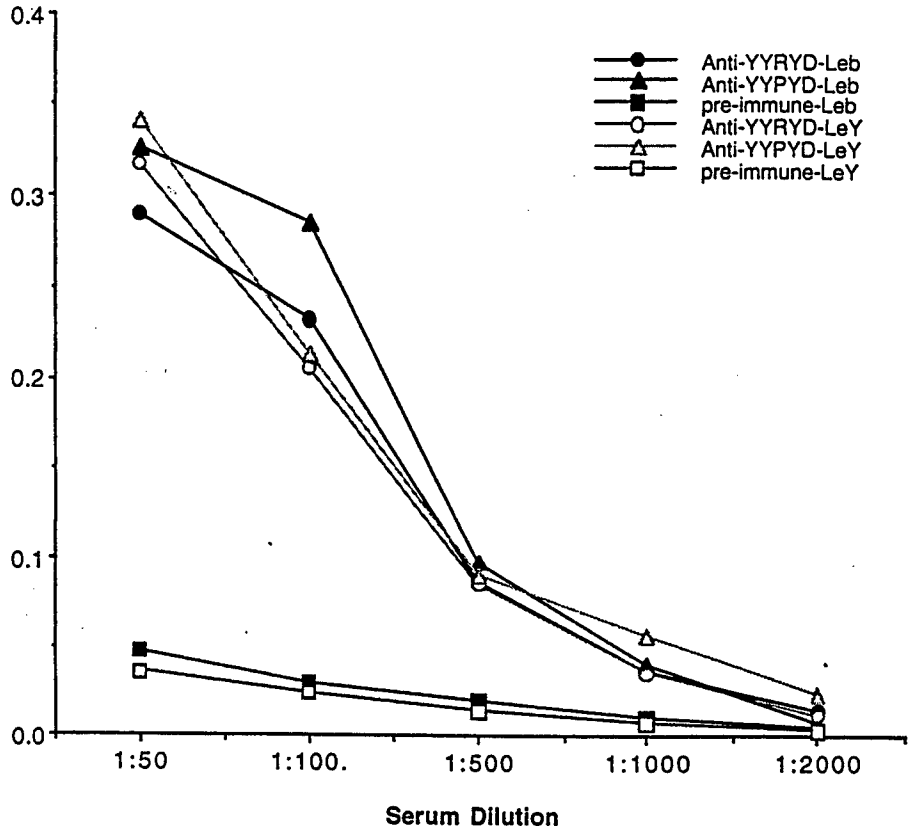

Fig. 3. Titration of the IgG portion of the peptide-proteosome derived anti-peptide sera with LeY and Leb.

multivalent or multiple antigen peptide forms provide increased selectivity or avidity for the polyvalent or clustered LeY form.

3.3. Distribution of serum reactivity

An important consideration in the development of synthetic surrogate immunogens is the reactivity of induced IgG to react with naturally expressed carbohydrate on the tumor surface. We have evaluated the ability of IgG elicited by peptide-proteosome, compared with IgM fractions induced by MAP forms of the same peptides, to bind to representative tumor cells as evaluated by FACS assay (Table 2). Positive control monoclonals were BR55-2 and ME361. Normal mouse sera (NMS) and sera generated against proteosome alone were also used as controls. Of interest was whether induced IgG or IgM serum reacts the same with LeY expressing cells. We found that serum from MAP peptide immunized mice displayed a higher mean fluorescence for MCF7 cells, than that induced by the proteosome formulation. For SKBR3 cells, both serum types reacted about the same. Both serum types displayed minimal reactivity with the normal breast cell line and murine fibroblast. Anti-YRY and anti-YPY sera reacted with WM793 cells but to different degrees. Melanoma cells have recently been shown to express sLeX and sLea (Ravindranath et al., 1997a). The WM793 cell line was found to be reactive with MAb FH-6. Our results suggest, that the respective serum is cross-reacting with sLeX on the melanoma cells, since we see some reactivity with this probe.

We extended our examination for cross-reactivity to include both normal tissue and tumor specimens. Rabbit serum to the YRY P2 peptide was chosen to screen normal tissues, because this peptide elicits sera that display reactivity for both MCP and LeY. Therefore, it may be perceived that this peptide might induce responses with broad reactivity for a variety of carbohydrates, expressed on the surface of human tissue. Thirty-eight fresh normal tissue samples, 33 paraffin-embedded normal tissue samples, 43 paraffin-embedded epithelial tumor samples including tumors of the colon, stomach, breast, lung, prostate, bladder and pancreas and 23 fresh epithelial tumor samples were examined and graded (Table 3). These results indicate that the generated rabbit sera display weak binding in the majority of normal samples, similarly observed for the LeY specific BR55-2 MAb. As expected, strong binding was observed in the majority of tumors examined. These data further suggest that the generated sera are minimally binding to normal tissues,

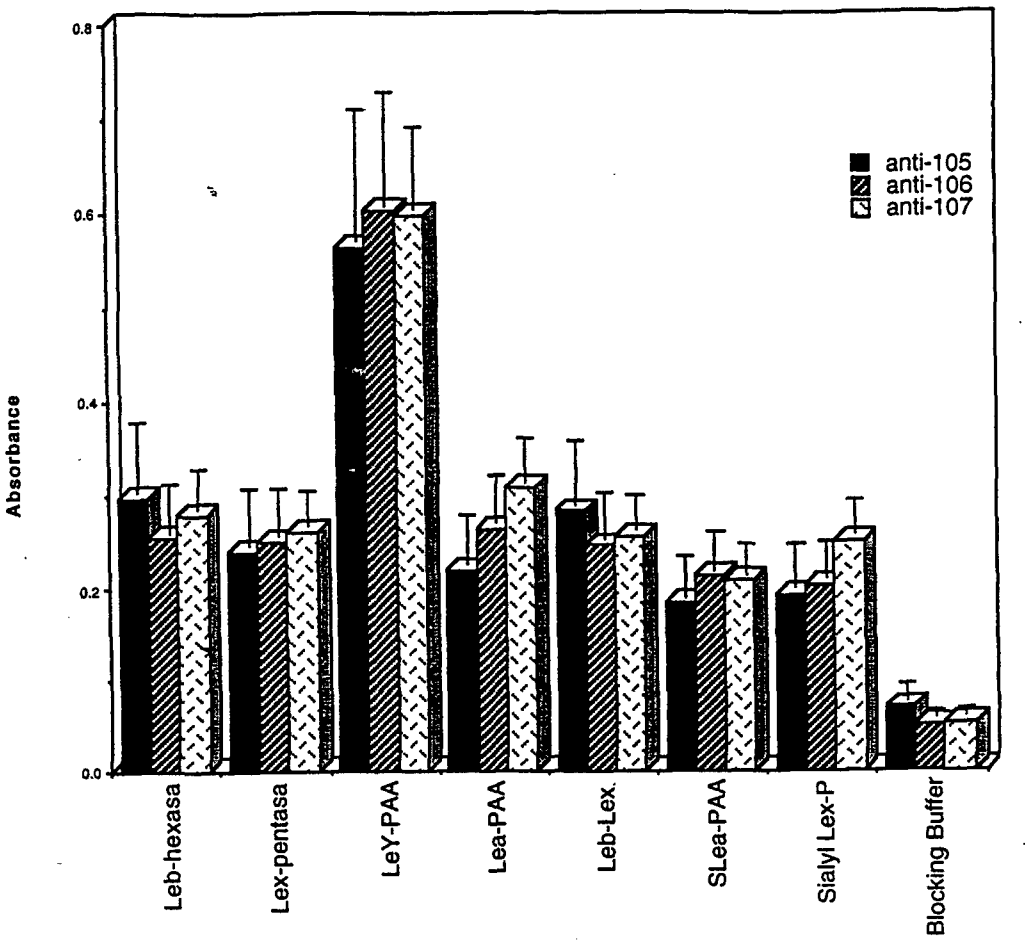

Fig. 4. Profile of cross-reactivity of anti-peptide MAP sera with Le carbohydrate probes.

Table 2
Binding of various anti-peptide sera to different cells as measured by FACS

Cell Lines	Anti P1 (YYPY)	Anti P2 (YYRYD)	Anti 1105 MAP	Anti 1107 MAP	ME361 (100 μg/ml)	BR55-2 (100 μg/ml)
SKBR5	60.0	86	ND	ND	3.1	59.0
MCF7	63/144[a]	54	150	176	5.4	352
SKBR3	240.6	275.6	240	250	3.2	235.6
HS578 Bst (normal breast)	17.8	19.9	18.6	18.4	ND	16.2
SKMEL-28	47.0	33.0	49.8	43.6	26	13.8
WM793	42.3	145.5	35.7	178.4	92.1	15.4
NIH3T3 Murine Fibroblasts	20.9	21.8	24.5	21.2	ND	15.3

Background fluorescence (mean fluorescence) associated with nonspecific mouse sera is 24.2 and 23.7 for SKBR3 and NIH 3T3 cells, respectively. ME361 is 14.0 and 10.0 for SKBR5 and MCF7. Background for the human melanoma line was on average 24.4. (final sera concentration: 1:50).
[a] Final dilution at 1:20.

while displaying strong binding to tumor tissues that over-express histo-blood group related carbohydrates. This data suggests that even though these sera react with common structural features of MCP and LeY, the presentation of related carbohydrate forms on tissues is affected by the carrier molecules to which they are attached as previously suggested (Saito et al., 1994) or the density of expressed carbohydrate is low.

Table 3
Summary of carbohydrate expression on human tissues

Tissue Type	Total	Sera Reactivity				BR55-2 reactivity			
		+++	++	+	−	+++	++	+	−
Normal tissues									
Stomach	5			1	4				5
Pancreas	7			2	5			2	5
Ovary	20			4	16			2	18
Breast	15			4	11			2	13
Lung	4			2	2			1	3
Heart	2				2				2
Prostate	12			2	10			3	9
Thymus	6			2	4			2	4
Tumors									
Breast	20	20				19	1		
Lung	11	9	2			10	1		
Ovary	20	17	3			17	3		
Pancreas	4	2	2			4			
Bladder	5	4	1			4	1		
Prostate	6	4	2			4	2		

Carbohydrate expression was determined by the avidin–biotin-immunoperoxidase method and scored according to staining intensity and abundance of immunostaining: +++ strong, ++ moderate, + weak, − negative. The numbers under total refer to numbers of individual samples from different individuals that were tested. The numbers in the body correspond to the number of samples that fall into specific categories of reactivity.

3.4. *Carbohydrate modification affects serum reactivity*

The ability of the peptides to mimic carbohydrate fragments or subunits on the cell surface is further observed in consideration of pretreating cells with neuraminidase, followed by serum binding (Figure 5). Treating SKBR3 cells with neuraminidase marginally decreased BR55-2 antibody binding to the cells, while marginally increasing reactivity with the anti-peptide sera (Figure 5a). This result indicates that some of the carbohydrates are sialylated (i.e. sTn) on the SKBR3 line and their removal may affect the conformational properties of some carbohydrates, exposing new epitopes recognized by the anti-sera. Treatment of the WM793 human melanoma line with neuraminidase significantly decreased ME361 recognition of these cells, consistent with the recognition of sialyl subunits on the GD2/GD3 antigen (Figure 5b). Significant increases in the mean fluorescence was observed for serum binding to WM793 cells. This increased binding is interpreted as exposing otherwise encryptic epitopes on the cell surface after sialic acid removal. The core structure for GD2 is GalNAcβ1→4Gal[3←2αNeuNAc8←2aNeuNAc]β1→4Glcβ1→1Cer and for GD3 (NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→1Cer). Presumably, the elimination of sialic acid results in exposure of GalNAcβ1–4Gal units associated with GD2 and Galβ1–4Glc units associated with GD3. Representative synthetic probes of these subunits are highly reactive with the anti-peptide sera. This data further suggests that anti-peptide sera target carbohydrate on the surface of tumor cells.

The carbohydrate reactivity of the anti-peptide sera was further characterized by immunoprecipitation of tumor cell lysates before and after treatment with tunicamycin (Figure 6). LeY epitopes are found on MUC-1 mucins, lower m.w. glycoproteins and glycolipids, as well as higher m.w. proteins like CEA and LAMP-1 (Pastan et al., 1991; Garrigues et al., 1994; Yin et al., 1996). BR55-2 immunoprecipitates (IP) neoproteins in the range between 43,000 and 200,000 kDa are found on SKBR3 cells (Figure 6a). This profile is similar to that observed for anti-LeY antibodies B1 and B3 (Pastan et al., 1991) and BR96 (Garrigues et al., 1994). IP with NMS indicates no reactivity within this molecular weight range (Figure 6a, panel A). Treatment of SKBR3 cells with tunicamycin for 2 h decreases neoglycoprotein reactivity with BR55-2, verifying the carbohydrate recognition of this antibody (Figure 6a, panel B). IP of SKBR3 cells with the anti-peptide sera indicates that the P1 and P2 anti-sera display an IP profile, similar to that of BR55-2 (Figure 6b). Strong reactive bands for P1 and P2 are in the range of 47–89 kDa, with weaker bands between 117 and 89 kDa. These IP bands correspond to LAMP-1, originally identified with the anti-LeY MAb BR96 (Garrigues et al., 1994). We found that treatment of cells with tunicamycin for 2 h decreases carbohydrate expression of neoglycoproteins reactive with the anti-sera (Figure 6c). However, unlike BR55-2 reactivity, the respective serum

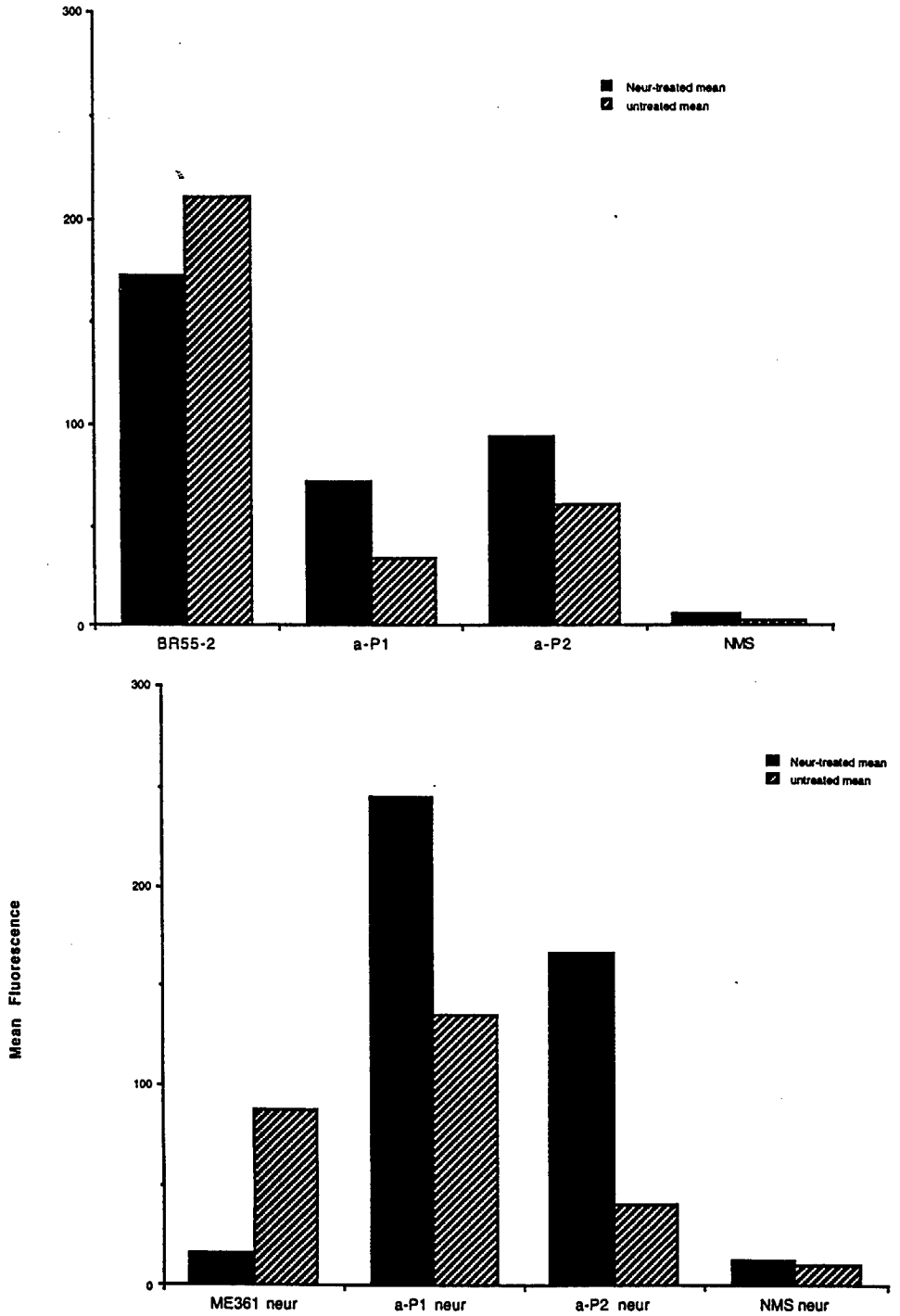

Fig. 5. Summary of FACs results for antisera binding to breast and melanoma cells, before and after neuraminadase treatment. (a) Pre- and posttreatment of SKBR3 cells. (b) Pre- and posttreatment of WM793 cells. The P1 sera correspond to the YYPYD motif and P2 to the YYRYD motif. Sera in both assays are diluted 1:100.

is reactive with suspected glycoprotein(s) around 47 kDa after 2 h. We observed that antisera directed toward the YRGD motif do not immunopercipitate any of the glycoproteins in the cell lysates pre- and posttreatment. This data further suggests that the anti-sera are reactive with carbohydrate epitopes on the cell surface, that is

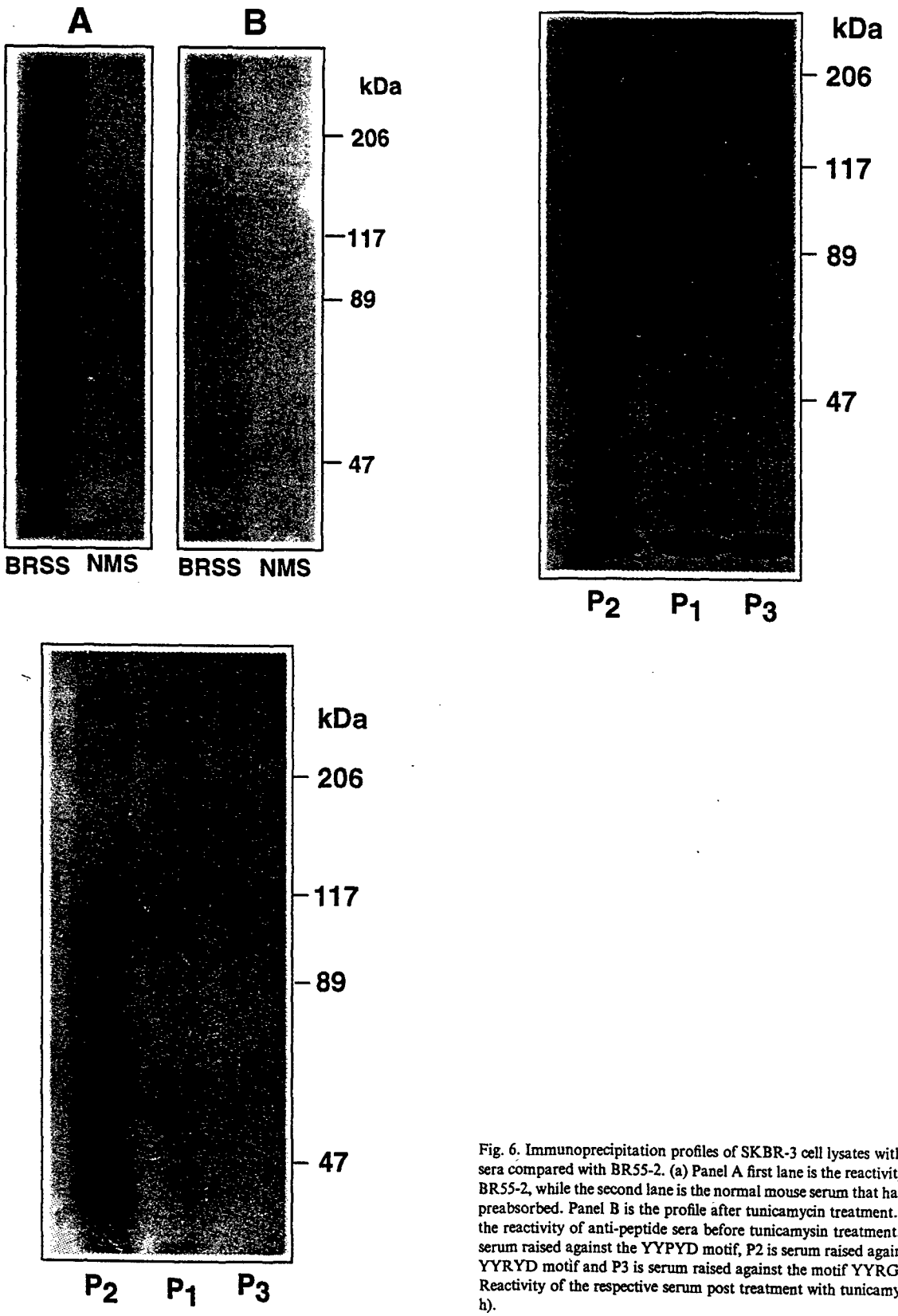

Fig. 6. Immunoprecipitation profiles of SKBR-3 cell lysates with antisera compared with BR55-2. (a) Panel A first lane is the reactivity with BR55-2, while the second lane is the normal mouse serum that has been preabsorbed. Panel B is the profile after tunicamycin treatment. (b) Is the reactivity of anti-peptide sera before tunicamysin treatment. P1 is serum raised against the YYPYD motif, P2 is serum raised against the YYRYD motif and P3 is serum raised against the motif YYRGD. (c) Reactivity of the respective serum post treatment with tunicamycin (2 h).

Table 4
Summary of complement dependent cytotoxicity results

Tumor	C1	P2	P1	LeY–PAA	ME361	BR55-2
SKMEl-28	3	32	10	4	10 (50 μg)	3 (100 μg)
SKBR3	6	90	86	20	10 (100 μg)	80 (100 μg)
MCF-7	3	66	92	26	5 (50 μg)	75 (100 μg)
WM793	5	28	9	2	63 (30 μg)	1 (100 μg)
OVAR-3	5	89	86	25	6 (50 μg)	80 (100 μg)

Values are averaged percent cytotoxicity. Final dilutions are 1:15 for sera. Monoclonal antibody ME361 and BR55-2 concentrations are per ml.

similar to that observed for anti-LeY monoclonal antibodies.

3.5. Tumor cell cytotoxicity

Anti-carbohydrate antibodies might mediate complement dependent cytotoxicity (CDC) better than cytotoxicity associated with various effector cells (Mayer et al., 1994). Consequently, we have examined CDC mediation of various sera raised either to peptides or against the multivalent LeY–PAA form. This form is immunogenic in mice, when adsorbed onto bacteria (*Salmonella minnesota*) (Vlasova et al., 1994). We examined the ability of the sera to mediate complement dependent cytotoxicity (CDC) of the SKBR3 and MCF7 human breast adenocarcinoma cell lines, the ovarian tumor cell line OVAR-3 and the human melanoma lines WM793 and SKMEL-28 (Table 4) compared to that of the LeY–PAA generated sera. Positive control antibodies were BR55-2, that mediates CDC of the adenocarcinoma lines and ME361 that mediates killing of WM793. Negative controls were pre-immune sera and an irrelevant peptide immunogen (C1).

In Table 4, serum to P1 and P2 showed an ability to mediate CDC of the LeY expressing human breast lines SKBR3 and the human ovarian line OVAR-3 similar to the positive control BR55-2 MAb. Serum against P2 showed diminished CDC activity for MCF7. Anti-P1 sera mediated CDC of the human melanoma lines close to nonspecific values, using control serum (C1) while anti-P2 sera displayed moderate CDC activity. While only several LeY expressing lines are shown in Table 4, these data indicate, that the functional response can be specific for carbohydrates, highly expressed on human tumors. The P1 reactive serum displays a clear preference for the adenocarcinoma cells, while P2 reacts slightly more with melanoma cells, which may be a function of their expression of sLeX. These data also suggest that despite the broad specificity for carbohydrate constituents by ELISA, the respective serum recognizes ubiquitous carbohydrate subunits differently when expressed on cells. These data indicate that a serum, generated to carbohydrate-mimicking peptides has the potential to recognize important tumor-associated antigens with a high degree of specificity.

4. Discussion

Immunization with simple synthetic Lewis antigen-conjugates, as observed with simple LeY-conjugates, only results in sera and MAbs, reactive with the immunizing antigen (Kitamura et al., 1994). In contrast, immunization with LeY expressing cell lines or multivalent LeY forms, yield MAbs that react with both synthetic carbohydrate forms and native carbohydrate configurations (Vlasova et al., 1994). One difference between these antigen sources is the distribution of LeY epitopes on the carrier — in the neoglycoprotein conjugates single LeY structures are substituted over the surface of the carrier protein, whereas in cells, LeY structures are substituted on a variety of carriers (e.g. glycoprotein) on some of which the epitope density is probably quite high. These results indicate that better ways to synthesize LeY immunogens that are reflective of naturally expressed LeY structures (Deshpande and Danishefsky, 1997; Kudryashov et al., 1998) or alternative ways to induce immune responses cross-reactive with native LeY are needed. One alternative is peptides as surrogate antigens.

Although peptides have been identified as mimotopes for saccharides, it was not certain that they could bind well enough to inhibit the binding of antibodies to nominal carbohydrate, nor induce cross-reactive immune responses to a nominal carbohydrate antigen. The identification of motifs like W/YXY that compete with carbohydrate for protein binding suggest that a particular peptide structure is required for polysaccharide mimicry (Evans et al., 1994). In contrast, other studies indicate that peptides appear to be specific for monoclonal antibodies used in peptide isolation from phage display and are not recognized by the same mechanism as their carbohydrate counterparts (Harris et al., 1997).

Our own studies have focused on determining whether the mechanism of mimicry of carbohydrates by peptides is structural and/or immunogenic. We have determined the molecular recognition properties of the core structure

of the tetrasaccharide LeY antigen to anti-LeY antibodies (Thurin-Blaszczyk et al., 1996; Murali and Kieber-Emmons, 1998) and observed that the LeY tetrasaccharide structure is similar to the core structure of MCP (Agadjanyan et al., 1997). A YRY-containing peptide elicits serum cross-reactive with MCP and basic constituents of lacto-*N*-neotetraose, that may in fact have contributed to protection in our earlier studies (Kieber-Emmons et al., 1997). The epitope NeuNAcα2–3Galβ1–4GlcNAc, which is a component of the neolactoseries antigen sLeX Fig. 1 has recently been identified as a *Neisseria* LOS component (Tsai et al., 1998). Consequently, evaluating responses to neolactoseries antigens will further define recognition specificities for antibodies directed to these biologically important carbohydrate forms.

ELISA screening with the anti-LeY MAb BR55-2 suggests that the YRY and WRY motifs better reflect a LeY-like topographical feature that is complementary to the BR55-2 combining site Fig. 2. These motifs are also reactive with the anti-sLeX MAb FH-6. Substitution of proline in the YXY motif diminishes the functional antigenic mimicry of the aromatic–aromatic motif for BR55-2 binding (Figure 2b). Of further interest is the lack of reactivity of BR55-2 and 15-6A for the K61223 peptide, that is a derivatized form of a peptide motif isolated from phage display with another anti-LeY antibody B3 (Hoess et al., 1993). We have recently put forth a structural explanation for the lack of reactivity of BR55-2 with the APWLY sequence, in which antibody contact residues for the peptide in CDR2 of the heavy chain of B3 are different from those in BR55-2 (Murali and Kieber-Emmons, 1998). These data further emphasize that antibodies discriminate peptides better than carbohydrate forms (Harris et al., 1997).

To test the immunological mimicry of the aromatic–aromatic containing motifs, mice were immunized with peptide-proteosome conjugates or as MAPs. The utility of MAP peptides is in their apparent advantage as immunogens (Rajadhyaksha et al., 1995; Kanda et al., 1994). MAPS have proved to retain all the immunological properties of an intact anti-id for example upon which the peptide was based (Kanda et al., 1994; Rajadhyaksha et al., 1995) and was found to be qualitatively similar and quantitatively superior to the linear monomeric 15-mer anti-id derived peptide (Rajadhyaksha et al., 1995) when MAPs contain a T cell epitope. Likewise, we observed a better degree of specificity for the LeY structure over other Le antigen forms (Figures 3–4). These results suggest that multivalent presentation of the peptide epitopes improves a motif specificity's, possibly mediated by avidity, similar to previous observations (Kanda et al., 1994; Rajadhyaksha et al., 1995).

Reactivity of sera, generated against the YRY motif with human normal and tumor specimens, indicate weak reactivity with normal tissues Table 3. Reactivity of a common carbohydrate epitope with different antibodies or ligands is highly dependent on the type of carrier glycosylceramide or carrier O-linked peptide (Saito et al., 1994) which can effectively restrict cross-reactivity with otherwise related carbohydrates expressed on normal tissues. The tumor association of a trisaccharide epitope constituent of histo-blood group antigens, Galb1–3GlcNAcb1–3Gal, indicates that this epitope is highly restricted to adenocarcinomas, as assessed by rabbit sera made to the trisaccharide coupled to BSA (Diakun et al., 1996). Our immunization studies also suggest, that if serum is reacting with normal tissues in mice and rabbits, there are no apparent adverse reactions.

Further evidence that the anti-peptide sera bind to carbohydrates on the cell surface comes from treatment of cells with neuraminidase Fig. 5 and from IP profiles Fig. 6. CDC results Table 4 indicate that the anti-peptide sera is specific for LeY-expressing cells. This might be the result of increased avidity for LeY. Some CDC activity is observed with WM793 cells. This may be due to the expression of sLeX on these cells, as evidenced from reactivity with the anti-sLeX antibody FH-6. Serum against the proteosome conjugated YRY motif reacts with sLeX.

The basis of peptide binding (antigenic mimicry) to anti-carbohydrate antibodies differs between antibodies, determined mainly by the antibody combining site (Harris et al., 1997). It is now evident that one antibody does not necessarily bind to a single antigen, but it may recognize antigens complementary to its combining site, even though the chemical components of the antigen may be very different, such as carbohydrates and peptides. The chemical nature of the mimicry between a carbohydrate and a mimicking peptide is not completely understood; however aromatic–aromatic and hydrophobic interactions appear to be critical chemical forces between carbohydrate-mimicking peptides and an antibody binding site (Young et al., 1997; Murali and Kieber-Emmons, 1998). Since antigen selection operates on functional, not structural conformations, the known difficulties met with antibody mimics, suggest that heterologous binding by unrelated molecular surfaces may be a common phenomenon in antigen–antibody interactions (Lescar et al., 1995). An understanding of the molecular principles that govern antibody specificity still remains a major challenge.

The present studies indicate that carbohydrate structures on tumor cells can be mimicked by peptides and suggests that appropriately constructed peptides may indeed be able to augment immunogenicity against carbohydrate antigens. While the peptides in this study do not elicit sera that are highly specific for LeY, we are optimizing LeY peptide mimotopes for this purpose. Peptide mimics may be designed as polymeric peptides mimicking more complex carbohydrates, or polyvalent vaccines may be produced, using heteropolymers of mim-

icking peptides. Mimicking peptides represent a new and very promising tool to overcome T cell independence and to increase efficiency of the immune response to carbohydrates. While peptide mimotopes hold promise in vaccine design, there are many issues related to their use as functional carbohydrate surrogates in designing efficacious vaccines. Some of these issues are fundamental questions that pertain to how mimicry comes about at the molecular level and some are application oriented, directed at elucidating important immunological mechanisms associated with vaccine efficacy. Nevertheless, peptides that mimic tumor-associated carbohydrates would be of importance as novel agents in adjuvant therapy (Shikhman and Cunningham, 1997).

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Peptide Mimotopes of Carbohydrate Antigens

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Abstract

Carbohydrate structures have been identified as significant antigens for bacterial, viral, and fungal pathogens as well as targets on human tumor cells. Many of these antigens are poorly immunogenic in humans, requiring extensive adjuvant sublimation. Although conjugate carbohydrate vaccines appear promising, there are limitations of using carbohydrate formulations. An alternative approach is to use surrogate antigens for some carbohydrates. We are developing peptides that mimic carbohydrates which might be further manipulated to induce responses that target biologically important carbohydrates expressed on pathogens and on tumor cells. We have shown that peptide mimotopes of carbohydrates induce immune responses to carbohydrate structures with in vivo and vitro functionality. Model systems include the Neisseria group C meningococcal polysaccharide; the histo-blood group-related antigens expressed on tumor cells; and mannose, sialyl, and histo-blood group-related carbohydrate epitopes expressed on human immunodeficiency virus.

Key Words

Peptide mimotopes
Carbohydrate antigens
Lewis Y
Vaccine
Adjuvants

Introduction

Carbohydrates play a very essential role in cell and immunobiology. They are involved in cell–cell communication, and participate in cell proliferation and differentiation (cell growth). Aberrant glycosylation of tumor cells is considered a basis for uncontrolled cell growth, invasiveness, and increases metastatic potential *(1,2)*. At the same time, carbohydrates are so ubiquitous in nature that they are shared among bacteria, viruses, and tumors. Some anticarbohydrate antibodies made against tumors crossreact with corresponding structures expressed on bacteria and viruses. Thus, one could conclude that both the pathophysiology of infection and neoplasia can be affected by the same or similar carbohydrate sub-structures.

The importance of carbohydrates as mediators of disease focuses attention on their formulation in vaccine development. Carbohydrates are T cell-independent (TI) antigens however, eliciting diminished immune responses toward

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them *(3)*. Since carbohydrate antigens are generally weakly immunogenic in humans, only short-lived IgM responses have been historically observed. In addition, some complex carbohydrates are difficult to synthesize or purify. Moreover, this latter aspect is further magnified if one considers that clustering of epitopes on neoglycoproteins must be emulated in the synthesis process, leading to multiple presentation or tandem repeats of the synthetic carbohydrate immunogen *(4)*.

The importance of adjuvant sublimation has been highlighted to offset the relatively weak immunogenicity of carbohydrate structures *(5)*. The general rationale is to try to convert them to T cell-dependent (TD) antigens. T-dependent protein vaccines can become a decisive factor in situations where the responding immune system is immature or suppressed. From experimental studies on animals, we know that the response to cell-dependent antigens matures earlier than the T-independent (TI) response to carbohydrate antigens, and that often a genetically or acquired insufficient immune system responds better to TD antigens than to TI antigens. This may be especially true for cancer patients, who are oftentimes immunocompetent to their own tumors and neonates under the age of 2.

Several different approaches have been employed to avoid the problems with carbohydrate vaccines, including the use of carbohydrate–protein conjugates *(5)* and anti-idiotypes *(6)* resembling a carbohydrate antigen. As a further alternative, we have been examining peptides as effective mimics of carbohydrates to induce antibody responses that target carbohydrate structures. Peptide antigens associate with major histocompatibility complex (MHC) and thus can be formulated to manipulate both Th1 and Th2 responses *(7)*. Mimicking peptides represent a new and very promising tool to overcome T cell-independence and to increase the efficiency of the immune response to carbohydrates. Peptides that mimic carbohydrate structures have significant advantages as vaccines compared with carbohydrate–protein conjugates anti-idiotypic antibodies. First the chemical composition and purity of synthesized peptides can be precisely defined and reproduced. Second, the immunogenicity of the peptides can be significantly enhanced by polymerization or addition of relatively small carrier molecules that reduce the total amount of antigen required for immunization. Third, peptide synthesis may be more practical than synthesis of carbohydrate–protein conjugates or the production of anti-idiotypes. Peptide mimics may be designed as polymeric peptides mimicking more complex carbohydrates, or polyvalent vaccines may be produced using heteropolymers of mimicking peptides. Subsequently, peptides that mimic tumor-associated carbohydrates would be of importance as novel agents for adjuvant therapy.

Here our efforts in the development of peptides that induce carbohydrate reactive immune responses is summarized. In particular, we have shown that peptides can immunologically mimic the meningococcal group C capsular polysaccharide *(8)*, and that this peptide, and related structures, can induce immune responses to carbohydrate epitopes on breast adenocarcinoma cell lines *(9)* and expressed on the envelope protein of human immunodeficiency virus HIV *(10)*.

Peptide Mimicry of a Bacterial-Associated Carbohydrate

The notion of using peptide mimics of carbohydrates to induce anticarbohydrate immune responses parallels the use of anti-idiotypic antibodies as immunogens. Some anti-idiotypes that mimic carbohydrates have been shown to induce anticarbohydrate immune responses in humans *(6,11)*. Some time ago, Westerink and colleagues defined an anti-

idiotypic antibody called 6F9 that mimics the major C-polysaccharide (MCP) of *Neisseria meningitis*. This antibody was sequenced and a YYRYD motif that was surface-exposed in the CDR3 of the heavy chain was identified as the putative MCP mimic *(8)*. We found the peptide sequence CARIYYRYDGTAY when complexed to proteosomes induces an anti-MCP antibody response in BALB/c mice that is protective in nature when peptide-immunized mice were challenged with a lethal dose of bacteria *(8)*. We designed the peptide by placing a YGG spacer at the N-terminus, preceded by a cysteine. To induce the formation of dimers or crosslinking between the peptides, we placed cystiene just before the tyrosin. To facilitate hydrophobic complexing to the proteosomes, we placed a lauroyl group at the very N-terminus.

BALB/c mice were hyperimmunized on a weekly basis with various peptide concentrations. The major Ig fraction on immunization was found to be IgM, with IgG coming up later. The sera were found to be protective in nature with a 100% survival rate across the dose range used. Immunization with proteosomes or saponin (QS-21) gave the same result. Thus, immunization with either proteosomes or with saponin provided the same level of protective immunity.

This peptide bears some homology with other peptides that mimic carbohydrates involving a Planar-X-Planar sequence motif (Table 1). All proteins which interact with α-amylase have WRY residues implicated in this interaction. Recently, Hoess et al. *(12)* identified an eight amino acid peptide expressed on phage capable of inhibiting Lewis Y carbohydrate-antibody binding, consisting of the sequence tract PWLY. A peptide which binds ConA has been shown to have a similar configuration, namely YPY *(13,14)*. These peptides demonstrate the preference of aromatic groups separated by an intervening residue.

Table 1. Peptide motifs that mimic carbohydrate structures

Peptide	Carbohydrate	Structure
YYPY (P1)	Mannose	Methyl-α-D-manno-pyranoside
WRY	Glucose	α(1–4)Glucose
PWLY	Lewis Y	Fucα1→2Galβ1→4 (Ficα1→3)GlcNAc
YYRYD (P2)	Group C poly-saccharide	α(2-9)sialic acid

All these sequences resemble the peptide we have identified as a mimic of the group C meningococcal polysaccharide. Peptides that mimic carbohydrates may follow similar "rules," employing functional and hydrophobic groups reminiscent of carbohydrate structures. The immunological presentation of the putative motifs (i.e., short or longer peptides, presentation in a helix or β bend) might mimic overlapping epitopes on otherwise different carbohydrate structures.

The homology displayed by the peptides in Table 1 suggests that the various carbohydrate forms mimicked by these peptides might display similar conformational features. This is further suggested in that sera and monoclonals made to the YYRYD, and YYPYD tracts crossreact with MCP on ELISA. In addition, antibodies against these motifs might also crossreact with tumor cells that display various subunit forms reflective of these carbohydrate subunits (Table 2). The sequence similarities among the putative motifs suggest that antibodies raised to this peptide set might crossreact with similar subunits expressed on what are otherwise dissimilar carbohydrate structures. Molecular modeling suggests that the Lewis Y (LeY) tetrasaccharide structure is similar to the core structure of MCP, suggesting that it is possible for antibodies to crossreact with these moieties *(10)*. Similarly, reactivity with LeY

Table 2. Survey of carbohydrate structures found on neoglycoproteins[a]

Number	Name	Structure
1	Lacto-*N*-fucopentaose I (H-type 1)	Fucα1-2Galβ1-3GlcNAcβ1-3galβ1-4(Glc)-APD
2	Lacto-*N*-fucopentaose II (Lea)	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD 4 | Fucα1
3	Lacto-*N*-fucopentaose III (Lex)	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)-APD 3 | Fucα1
4	Lacto-*N*-difuconeohexaose I (Ley)	Fucα1-2Galβ1-4GlcNAcβ1-*O*-APE 3 | Fucα1
5	Lacto-*N*-difucohexaose (Leb)	Fucα1-2Galβ1-3GlaNAcβ1-3Galβ1-4(Glc)-APD 4 | Fucα1
6	Maltose	Glcα1-4Glcα1-*O*-PAP
7	Lactose	Galβ1-4Glcα1-*O*-PAP
8	Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD
9	Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)-APD
10	Lacto-*N*-hexaose	Galb1-4GlcNAcβ1 | 6 Galβ1-4(Glc)-APD 3 | Galb1-4GlcNAcβ1
11	Lacto-*N*-neohexaose	Galb1-4GlcNAcβ1 | 6 Galβ1-4(Glc)-APD 3 | Galβ1-3GlcNAcβ1
12	Melibiose	Galα1-6Glcβ1-*O*-PAP
13	Cellobiose	Glcβ1-4Glcβ1-*O*-PAP
14	A-trisaccharide	GalNAcα1-3Galβ1-*O*-APE 2 | Fucα1
15	B-trisaccharide	Galα1-3Galβ1-*O*-APE 2 | Fucα1
16	A-tetrasaccharide	GalNAcα1-3Galβ1-4(Glc)-APD 2 | Fucα1
17	A-heptasaccharide	GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD 2 4 | | Fucα1 Fucα1
18	2-Fucosyllactosamine (H-type 2)	Fucα1-2Galβ1-4GlcNAcβ1-*O*-APE

[a]This tabl

19	Gangliotetraose	Galβ1-3GalNAcβ1-4Galβ1-4(Glc)-APD
20	T-antigen	Galβ1-3GalNAcα1-*O*-APE
21	3'-Sialylactose	Neu5Acα2-3galβ1-4(Glc)-APD
22	6'-Sialylactose	Neu5Acα2-6Galβ1-4(Glc)-APD
23	Sialyllacto-*N*-tetraose a (LSTc)	Neu5Aα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD
24	Sialyllacto-*N*-tetraose b (LSTc)	Neu5Acα2 | 6 Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD
25	Sialyllacto-*N*-neotetraose c (LSTc)	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(glc)-APD
26	Sialylated lacto-*N*-fucopentatose II (SLea)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD 4 | Fucα1
27	Sialylated lacto-*N*-fucopentatose III (SLex)	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)-APD 3 | Fucα1
28	Disialylated lacto-*N*-tetraose	Neu5Acα2 | 6 Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD
29	Chitotriose	GlcNAcβ1-4GlcNAcβ1-4(GlcNAc)-APD
30	Man3GlcNac	Manα1-2Manα1-3Manβ1-4(GlcNAc)APD
31	Man2GlcNAc	Manα1-3Manα1-4(GlcNAc)-APD
32	Biantennery-octasaccharide	Galβ1-4GlcNAcβ1-2Manα1 | 6 Manβ1-4(GlcNAc)-APD 3 | Galβ1-4GlcNAcβ1-2Manα1
33	Globotriose	Galα1-4Galβ1-4(Glc)-APD
34	Globotetraose	GalNAcβ1-3Galα1-4Galβ1-4(Glc)-APD GlcNAcβ1-6 | 1
35	GlcNAcT	Galβ1-3GalNAcα1-*O*-APE
36	Difucosyl Lea/Lex	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-*O*-APE 4 3 | | Fucα1 Fucα1
37	Difucosyl Lex	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-*O*-APE 3 3 | | Fucα1 Fucα1
38	Trifucosyl Lex	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-*O*-APE 3 3 3 | | | Fucα1 Fucα1 Fucα1
39	LacNAcT	Galβ1-4GlcNAcβ1-6 | 1 Galβ1-3GalNAcα1-*O*-APE

[a]This table emphasizes the ubiquitous nature of carbohydrate subunits.

might extend to Leb (Table 2) *(15)*. Superposition of LeY and Leb structures indicates that in spite of the change of glycosidic linkage from β1-3 to β1-4 in the type 1 and 2 chains, resulting conformational features of the respective sugar moieties are still shared forming a common topography *(15)*. The only effective difference is the position of the N-acetyl and hydroxymethyl groups projected on opposite sides of the type 1 and 2 difucosylated structures.

To test this idea, BALB/c mice were immunized with peptide–proteosome conjugates representative of the motifs YRY (P2) and YPY (P1). Sera were collected 1 wk after the last immunization, pooled, and tested for reactivity with LeY and Leb. We found that sera developed from immunizations with the putative P1 and P2 motifs react with the two multivalent probes, with the IgG reactivity going out to 1:2000 (Fig. 1). These data suggest that the sera are reacting with the common topography of the two synthetic probes as suggested from our molecular modeling studies.

Peptide Mimicry of Tumor-Associated Carbohydrate Antigens

Cell-surface carbohydrates undergo dramatic changes in cancer. Aberrant glycosylation in tumors relative to their normal counterparts represents a phenotypic feature associated with different human malignancies *(1,2)*. This phenomenon has been demonstrated repeatedly at frequencies higher than those of oncogenes and suppressor genes in various tumors. Aberrant glycosylation influences tumor progression, since cells acquire competence for metastasis and faster clonal growth via newly synthesized carbohydrate structures. The expression of branched and sialylated complex-type N- and O-linked oligosaccharides in malignant tumor cells appears to be directly associated with metastatic potential. Metastases may or may not be predicted based on the expression of these antigens *(16,17)*. Nevertheless, the expression of complex carbohydrates has been shown to predict unfavorable prognosis in breast and other cancers. These observations indicate that carbohydrates play an important role in cancer biology and are prominent targets for immunotherapeutic strategies to treat cancer.

Several types of altered glycosylation involving lacto series and related blood group structures have been described in a variety of human cancers, including lung, ovarian, pancreatic, colorectal, and breast (Table 2). These include:

1. Enhanced expression of GlcNAcβ1→6Manβ1→6 units appears to correlate with progression of human mammary carcinoma *(18,19)*.
2. The T, Tn, and sTn structures, Galβ1→3GalNAcβα, GalNAcα, and NeuAcα2-3GalNAcα, respectively, are powerful histologic markers in diagnosis and prognosis, occurring as surface antigens on most primary human breast carcinomas and their metastases, and are able to elicit both humoral and cell-mediated immunity *(20–22)*.
3. Human breast carcinomas express the histo-blood group antigen H on the globoside backbone Fucα1-2Galβ1-3GalNAcβ1-3Galα1-Galβ1-4Glc-Cer *(23)*.
4. Sialylated derivatives of type 1 and 2 Lewis antigens, such as sialyl-Le^a (SA-Le^a) NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glcβ1→R and sialyl-Lex (SA-Lex) NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→R are observed more frequently than their respective non-sialylated forms on breast adenocarcinomas *(24)*.
5. It has been observed that the expression of sialyl-dimeric Lex increases as the metastatic potential of colorectal carcinomas increases, and is related to the progression of colorectal cancer *(25,26)*.
6. The accumulation of α-fucosylated derivatives of lactoseries type 1 and 2 structures, such as LeY, Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→R, H-2, and Le^b, is closely

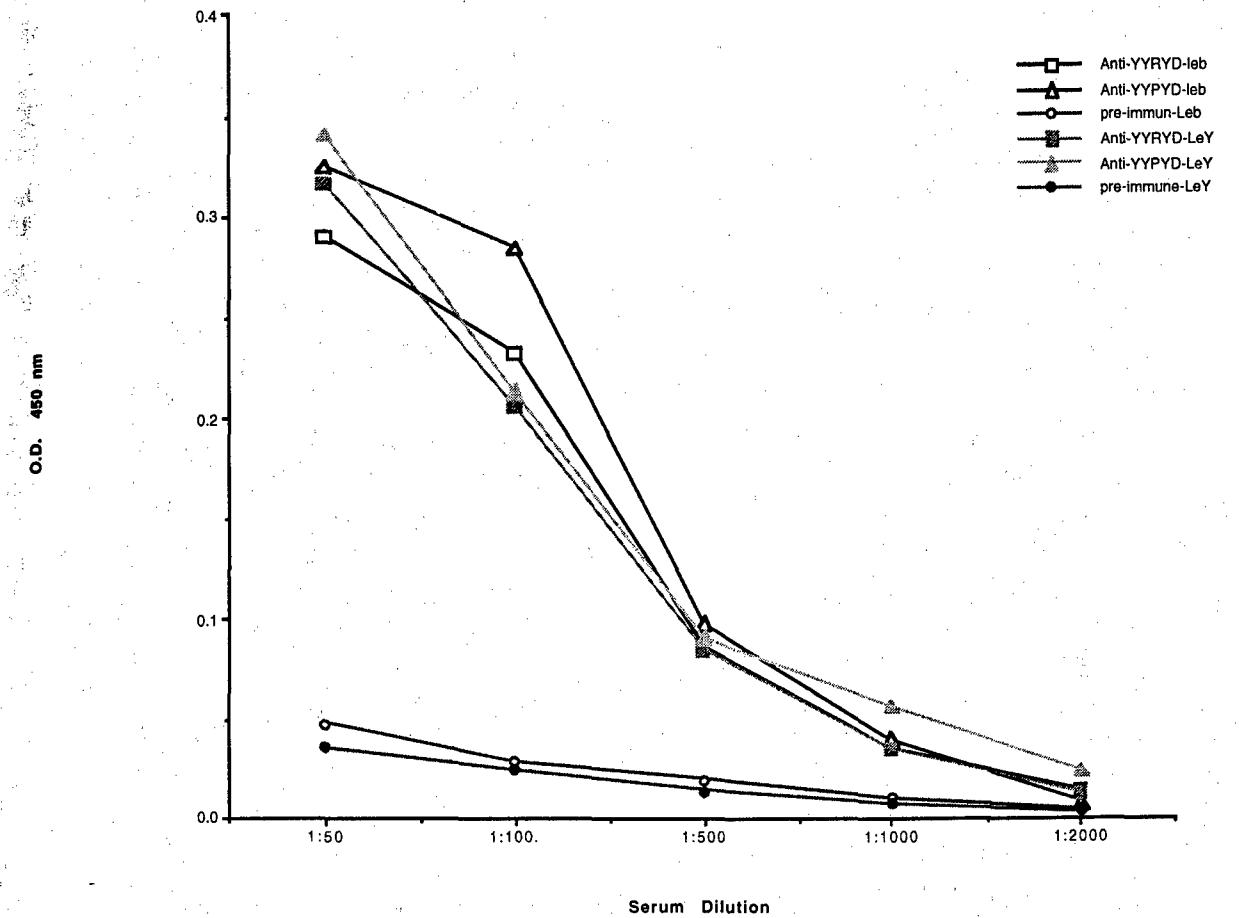

Fig. 1. ELISA reactivity of antipeptide sera with LeY and Leb. LeY and Leb probes are 30-kDa polyacrylamide forms (LeY-PAA, Leb-PAA) (Glycotech Inc.). Probes did not react with preimmune sera, proteosome-generated antisera, and control peptide immunization (latter data not shown).

associated with adenocarcinoma development and correlates inversely with the survival of patients with primary lung adenocarcinoma *(27–31)*. These forms also accumulate on colorectal carcinomas *(32)*.

Very few groups are investigating carbohydrate-based vaccines or carbohydrate-based immunotherapy. One major reason for this is that carbohydrate antigens are expensive and very difficult to synthesize. Further, expression of tumor-associated carbohydrate antigens is by no means specific to tumors. Crucial issues are expression of antigen density, multivalency, reactivity threshold of antibody binding, and efficient production of antibody having a strong complement-dependent or TD cytotoxic effect on tumor cells without damage to normal tissues. Studies on cancer vaccine development depend on many factors for success, which include:

1. Selection of carbohydrate epitopes.
2. Design and assembly of epitopes coupled to macromolecular complex as an efficient immunogen.
3. Establishment or availability of a good animal model.
4. Evaluation of immune response in animals and tumor rejection without damage to normal tissues.
5. Careful clinical application.

Since carbohydrate antigens are generally weakly immunogenic in humans, only short-lived IgM responses have been historically observed. The importance of adjuvant subli-

mation is highlighted in such studies to offset the relatively weak immunogenicity of carbohydrate structures *(5,21,33–40)*. Although these results show promise for active specific immunotherapy with carbohydrate formulations, they point out the limitations of this approach. These include: difficulty in antigen purification or synthesis, the utility of carbohydrate carrier–protein coupling strategies, which might prove to be impractical for broad application, and the lack of persistent high-titer cytotoxic antibodies in many patients. In general terms, with regard to a major objective of carbohydrate vaccines, i.e., generation of cytotoxic antibodies, variability in patient response and lack of persistence of high-titered IgM cytotoxic antibodies in many patients immunized with carbohydrate conjugates are problems that remain to be solved *(41)* and emphasize the still current limitation of this type of approach.

Results demonstrate that although vaccines containing TF or sTn-KLH conjugates plus immunological adjuvants Detox and especially QS-21 induce high IgM and IgG antibody titers in patients against the respective synthetic disaccharide epitopes, when tested against natural antigens expressing these disaccharide epitopes, IgM antibodies showed weak to moderate reactivity, whereas IgG antibodies were almost totally unreactive *(42)*. On the basis of these results, modifications of synthetic TF and sTn epitopes to identify those that induce IgM and IgG antibodies that are more reactive with these antigens as they are expressed on tumor mucins is warranted *(4,37)*. This general trend of nonreactiveness with naturally expressed carbohydrate epitopes on tumor cells is also observed for LeY *(43)*. These difficulties might be further magnified by considering that neoglycoconguates in which the carbohydrate structures are clustered together might make better immunogens *(4,42)*. It is argued that such structures would more closely resemble the arrangement of carbohydrate epitopes on the cell-surface—either glycolipid aggregates, multiantenary N-linked chains, or clustered O-linked oligosaccharides on mucins. Despite this widely held view, there is little experimental support for this concept. The most convincing evidence for this concept comes from examining the specificity of MAb raised to cells or other sources. For example, in a recent study, it was shown that a clustered neoglycoprotein antigen consisting of STn substituted on a triserine peptide, which in turn is coupled to KLH, gave an antibody response in mice that recognized clustered epitopes and not linear forms of STn *(4)*. Despite these promising results, synthetic routes for developing clustered carbohydrate epitopes are not straightforward.

Another approach for augmentation of carbohydrate immunity is the potential use of anti-idiotypic (anti-Ids) antibodies *(44)*. This approach requires that a polypeptide immunoglobulin variable region can mimic a carbohydrate determinant, providing a surrogate immunogen *(45,46)*. The immunologic utility of anti-Ids rests on protein mimics having the capacity to induce increased titers following booster immunization. Investigations centered on the development of anti-idiotypic and anti-anti-idiotypic antibodies to a structurally defined carbohydrate Ag, 3-O-α-L-fucopyranosyl-β-D-galactopyranoside (Fuc α 1——3Gal) (found on tissues from areas of benign and malignant disease of the colon and breast) have found that the exquisite specificity of binding of the original Ab1, with the antibody–Ag reaction requiring both fucose and galactose and the α-anomeric 1——3 linkage, was repeated with the anti-anti-idiotypes antibodies. This information indicates that although antigenic mimicry of anti-idiotypes for Ag is accomplished using amino acids in place of sugars, the specificity pattern can be precisely

reproduced *(45)*. For example, the notion that anti-Id-based vaccines mimicking carbohydrates might prove useful has come from analysis of HAMA and anti-idiotypic levels of patients that have been administered anti-ganglioside antibodies *(11,47)*. The same might hold true for T and Tn antigen. Idiotypes of pre-existing human anticarcinoma anti-T (Thomsen-Friedenreich) and anti-Tn antibodies have been observed *(48)*. Anti-Id responses in patients that mimic sTn have have also been noted on administration of the anti-Tag72 antibody B72.3 *(49)*. Preliminary results suggested that patients with high Ab2 serum concentrations had better survival rates compared to those where low or no Ab2 serum levels were detected *(49)*.

The molecular basis of how antibodies that mimic carbohydrate tumor-associated antigens (TAA) to induce tumor-specific immune responses is not, however, well characterized. Animal studies on network manipulation suggest that in tumor hosts, the concomitant idiotypic network response controls tumor growth and progression *(47,50–52)*. Anti-idiotypic probes have been used to delineate patterns of idiotope expression that might correlate with immunity against tumors *(11, 47,48)*. Certain idiotopes are found in animals that reject tumors and others are high in animals in which the tumors grow unrestrained. Since the growth of tumors does not necessarily correlate with the titer of antibodies directed against TAA, the effective tumor immunity could be mediated by T cells *(53)*. In light of the postulate that antibodies and T cells play a role in controlling tumor growth, surrogate antigens should be evaluated as a potential modulator of both humoral and cellular immune responses against TAA. Thus, the idiotypic network, demonstrated in serum by anti-idiotypic probes, is not necessarily involved in the regulation of the humoral antitumor response, but could also be acting on T effector cells, augmenting responses directed against tumor cells. One suggestion for T cell-mediated immunity is through the formation of immune complexes of Ids and anti-ids on tumor surfaces that appear to be the targets of T cells augmenting antibody-dependent cytotoxicity (ADCC). Such an explanation has been described as a mechanism for targeting the siaylated seal antigen on breast tumors *(54)*.

The notion of using peptide mimics of carbohydrates to induce anticarbohydrate immune responses parallels the use of anti-idiotypic antibodies as immunogens. Peptides that mimic carbohydrates might be used to augment naturally available immunoglobulins to tumor antigens. It is now also clear that humans with cancer have, in their draining lymph nodes, precursors of cytotoxic T cells that can be stimulated in vitro to react against their tumors *(55)*. Peptide formulations might trigger such precursors. Subsequently peptides that mimic tumor-associated carbohydrates would be of importance as novel agents for adjuvant therapy.

An important consideration in the development of the synthetic surrogate immunogens is the reactivity of the induced IgG sera to react with naturally expressed carbohydrate on the tumor surface. We have evaluated the ability of the IgG portion of sera to peptides containing the respective P1 and P2 putative motifs to bind to representative tumor cells as evaluated by FACS assay (Table 3). Positive control monoclonals were the anti-LeY antibody BR55-2 and the anti-GD2/GD3 ganglioside antibody ME361. Normal mouse sera (NMS) and sera generated against proteosome alone were also used as a controls. We found that murine sera elicited against these peptides bind to human breast carcinoma and melanoma cells. We also tested sera raised to a YYRYD-related motif, YYRGD (referred to as P3). The RYD sequence has been shown to be a mimic for the adhesion motif RGD *(56)*, and its conformational properties correlate

Table 3. Binding of various antipeptide sera to different cells ss measured by FACS[a]

Cell line	Anit P1, YYPY	Anti P2, YYRYD	Anti P3, YYRGD	BR 55-2
SKBR5	60.0	86	88/111[b]	59.0
MCF7	63/144[b]	54	63	155
SKBR3	240.6	275.6	166.7	235.6
HS578 Bst (normal breast)	17.8	19.9	22.4	16.2
WM793	145.5	42.3	172.44	15.4
NIH3T3 Murine fibroblasts	20.9	21.8	41.7	ND

[a]Background fluorescence (mean fluorescence) associated with nonspecific mouse sera is 24.2, and 23.7 for SKBR3, and NIH 3T3 cells, respectively. ME361 is 14.0 and 10.0 for SKBR5 and MCF7. Background for the human melanoma line was on average 24.4 (final sera concentration: 1:50).

[b]Final dilution at 1:20.

with bioactive RGD compounds *(57)*. The antipeptide sera to the YRGD motif was found to bind to both the human breast cancer cell lines and the human melanoma line WM793, displaying similar mean fluorescence as the other two antipeptide sera, with all three antipeptide sera displaying preferential binding to SKBR3. Most importantly, none of the sera reacted with normal breast cells indicating that these sera only react with cells that express high levels of respective carbohydrate antigens. This observation suggests that peptides can induce IgG responses that react selectively with tumors that express cell-surface carbohydrates. These results are in contrast with those using synthetic formulations of LeY in which anti-LeY sera was nonreactive with LeY expressing tumor cells.

We have also examined the ability of the sera to mediate complement-dependent cytotoxicity (CDC) of the SKBR3 and MCF7 human adenocarcinoma cell lines and the WM793 human melanoma line (Table 4). Positive control antibodies were BR55-2, which mediates CDC of the adenocarnioma lines, and ME361, which mediates killing of WM793. As expected, we observed that the anti-Lewis Y antibody BR55-2 mediates CDC of the adenocarcinoma cells at high antibody concentrations, approaching 80% cytotoxicity. ME361 did not mediate CDC of these lines even at 100 μg/mL concentrations. ME361 did mediate killing of WM793. We observe that the three antipeptide sera mediate CDC of SKBR3, approaching 80% at a dilution of 1:10, with diminished activity for MCF-7. The YYPYD sera did mediate MCF-7 killing similar to that of BR55-2. Although the antise was observed to bind to the human melanoma WM793 line assessed by FACS (Table 3), only marginal CDC was observed. These data suggest that the peptides can induce immune responses that target tumor cells, mediating their killing in vitro.

Peptide Mimicry of Carbohydrate Epitopes on HIV

Cancer-related mucin-type carbohydrate epitopes, principally mannose and sialo-syl residues, are also expressed on gp120 and gp160 of HIV. Carbohydrate epitopes are highly conserved among HIV isolates and as such, are considered potential targets for group-specific vaccine development. Anti-

Table 4. Summary of complement-dependent cytotoxicity[a]

Tumor	Preimmune	YYRYD	YYPYD	YYRGD	ME361	BR55-2
SKBR3	7	80	90	80	10 (100 μg)	80 (100μg)
MCF-7	1	29	66	19	5 (50 μg)	75 (100 μg)
WM793	8	9	9	22	63 (30 μg)	1

[a]Values are averaged percent cytotoxicity. Final dilutions are 1:10 for sera. MAb concentrations are per milliliter.

carbohydrate antibodies directed toward these and other carbohydrate epitopes are known to neutralize HIV-1 infection with cell-free virus. Antibodies directed to the Tn (GalNAc-Ser/Thr) or sialo-syl-Tn(NeuAc-GalNAc-Ser/Thr) antigen, occurring as a surface antigen on most primary human breast carcinomas and their metastases, inhibit HIV infection and syncytium formation *(58–60)*, as well as interacting with breast adenocarcinoma cells *(20,36)*. The tumor-associated histo-blood group antigen Lewis Y (Galβ1→4[Fucα1→3] GlcNAcβ1→3Galβ1→4Glcβ1→R) is also expressed on HIV-infected human T cells and monocytes *(61,62)*, as well as released HIV particles, whereas uninfected counterparts are Lewis Y-negative. Anti-Lewis Y and anti-Lewis X antibodies and lectins exhibit HIV neutralization and block syncytium formation *(63–65)*. Ganglioside expression on human T cell lymphotropic virus type I-HTLV-I-infected cells have also been determined by using a panel of MAbs originally generated against melanoma cells *(66)*.

Antisera to peptides representative of the YXY motif found to mimic carbohydrate structures associated with the lipo-oligosaccharides were examined for their ability to bind to glycosylated HIV envelope protein and to neutralize HIV in syncytia neutralization assays *(10)*. Peptides containing motifs mimicking methyl-α-D-mannopyranoside (YYPYD) and sialo-syl (YYRYD) residues were synthesized and complexed to proteosomes. A third motif, YYRGD, which modifies the YXY sequence tract, was also examined. We found that humoral immune responses can be induced in mice following immunization with respective peptide–proteosome complex that bound internal glycosylated glycoproteins gp140 and gp 120 from two diverse HIV-1 isolates (MN and SF). Generation of antibodies was not Ir-gene dependent, because at least two different strain of mice BALB/c (H-2^d) and C57Bl/6 (H-2^b) responded equally to the peptides. Importantly, the same antisera did not bind nonglycosylated gp120 from HIV-I/SF. Anti-YYPYD and YYRYD, but not Anti-YYRGD sera also displayed biological activity as was shown by neutralization of HIV-I/MN and HIV-I/3B cell-free infection of target cells. This neutralization was as good as human anti-HIV sera. These results indicate that peptide–proteosome complexes are perhaps MHC-unrestricted and that these peptides potentially convert otherwise T cell-independent polysaccharide epitopes to T cell dependent peptide epitopes, which direct immune responses toward carbohydrates. This approach provides a novel strategy for the further development of an HIV vaccine.

Summary

The present chapter provided salient results from our studies of peptides that mimic carbohydrate structures. We observe that peptides can mimic both binding and immunological events. These studies further substantiate that antibodies to common carbohydrate subunits found on bacteria and tumor cells can also bind

viral glycoprotein. Our results also support the premise that the use of peptide antigens, which are mimics of carbohydrates, is an alternative vaccine strategy for polysaccharide antigens resulting in an appropriate response. We have found that mimicry is the result of conformation or electrostatic properties that are shared among carbohydrate subunits. Peptide motifs can emulate sugar functional groups and their spatial arrangements: hydroxyl groups, planar groups, and backbone hydrogen bonding. Peptides that mimic carbohydrates may follow identical rules, but there may be some differences. Although the degree of carbohydrate reactivity is broad, these studies indicate that carbohydrate structures can be mimicked by peptides and suggests that appropriately constructed peptides may indeed be able to augment immunogenicity against carbohydrate antigens. Peptide mimics may be designed as polymeric peptides mimicking more complex carbohydrates, or polyvalent vaccines may be produced using heteropolymers of mimicking peptides. Mimicking peptides represent a new and very promising tool to overcome T cell independence and to increase efficiency of the immune response to carbohydrates. Subsequently, peptides that mimic tumor-associated carbohydrates would be of importance as novel agents for adjuvant therapy.

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Vaccination with a carbohydrate peptide mimotope promotes anti-tumor responses

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Abstract

Many human carcinomas overexpress the Lewis Y (LeY) and a sialylated homologue Lewis X (sLeX) blood-group epitope. In an attempt to overcome the problems that arise from the T cell-independent immune response induced by carbohydrate antigens we have examined the immunogenicity of peptide mimotopes of these antigens in mice. Immunization of groups of mice with these peptides as multiple antigen peptide forms, together with the immunological adjuvant QS21, showed that the formulations were efficient for eliciting IgM antibody responses to naturally occurring forms of the antigens carried on mucins and glycolipids. These antibodies were cytotoxic to a human breast cancer cell line expressing LeY (MCF-7) in the presence of human complement. It was observed that the peptide mimotopes could prime for memory responses directed toward tumor cells expressing LeY. Vaccination with these peptides mediated tumor rejection of sLeX expressing Meth A sarcoma in vivo in a murine model. These experiments suggest that peptide mimotopes of the LeY tumor associated carbohydrate antigen and QS21 adjuvant could be considered as an immunogenic therapeutic vaccine in carcinoma patients in the minimal residual disease setting.

Introduction

Tumor-associated carbohydrate epitopes have shown promise as vaccines for active specific immunotherapy of cancer [1]. There are several aspects, however, that hamper vaccine or adjuvant therapy targeting carbohydrates. Carbohydrate antigens are typically poorly immunogenic, very difficult to purify in large quantities, difficult to synthesize, and usually induce mostly short-lived IgM type antibodies in a vaccinated host without long lasting immunity. Most carbohydrate antigens belong to the category of T cell-independent (TI) antigens that reflect their inability to stimulate MHC class II dependent T cell help [2]. As a consequence, carbohydrates are not capable of induction of a sufficient anamnestic or secondary immune response. In an attempt to overcome the problems that arise from the TI immune response induced by carbohydrate antigens, vaccine strategies have focused on development of polysaccharide-protein conjugates. The difficult steps in this approach are the purification of the carbohydrate and the loss of immunogenicity of the carbohydrate moiety during coupling to an immunologic protein carrier. Carbohydrate synthesis may diminish the problems associated with antigen purification, but remains a limited solution due to the overall difficulties of carbohydrate chemistry. Moreover, carbohydrate-conjugate vaccines differ chemically and immunologically. In addition, T cell help directed toward carbohydrate-conjugates are not carbohydrate specific. These properties translate into fundamental differences in antibody quality elicited by different carbohydrate-conjugate vaccines.

To overcome these drawbacks, we have initiated a program to develop peptides that mimic the antigenic properties of carbohydrates that may be further manipulated to trick the immune system into targeting tumor expressed carbohydrates. The concept of mimicry holds enormous promise; a molecule that mimics a given antigen by eliciting a similar immune response is potentially useful as a vaccine, and is defined as a mimotope [3]. Peptide mimotopes might help elicit longer lasting immune responses to tumors providing immunological memory related to vaccine composition, form, and delivery. Memory responses could thwart repeated presentation of metastases as a consequence of maintenance of high levels of circulating antibodies.

To develop rational design concepts to enhance carbohydrate cancer vaccine efforts, we have focused on the development of peptide mimotopes for the blood group-related neolactoseries carbohydrate structures as a model system. The neolactoseries structures Lewis X (LeX), sialyl-LeX (sLeX), Lewis a (Lea), sialyl-Lea (sLea) and Lewis Y (LeY) are examples of terminal carbohydrate structures related to tumor prognosis expressed on a variety of human tumors [4-6]. Here we demonstrate that peptides that mimic neolactoseries structures are particularly effective for induction of antibody responses in mice that react with human tumor cells and mediate their killing in the presence of human complement. Furthermore, the peptide mimotopes are effective as priming agents required for longer lasting memory responses and in diminishing tumor growth in tumor challenge studies.

Results

Immunogenic mimicry of peptide motifs

The neolactoseries structures LeY, LeX, sLeX, Lea, sLea, and Leb all share a common epitope topography [7-9]. Multiple antigen peptides, shown in Table 1, generate serum that is cross-reactive with the common topography of the neolactoseries structure, yet displays a higher avidity for LeY [10-12]. These anti-peptide serum antibodies are primarily of IgM isotype that are specific for neolactoseries expressing cell lines and human tumors, while displaying little reactivity with non-neolactoseries expressing cell lines and human tissues [12]. Anti-peptide serum binding is observed to a variety of human LeY expressing tumor lines that include human breast carcinoma lines SKBR3 and MCF7, and the LeY-sLeX expressing human prostate line PC-3 as assessed by FACs analysis (Table 2). Serum is also cross-reactive with the murine fibrosarcoma cell line Meth-A that expresses sLeX. Minimal reactivity is observed for serum with the LeY negative human melanoma line SK-MEL-28 which is GD3 positive and LeY negative. Some cross-reactivity with this cell line has been observed previously with serum raised to a LeY-conjugate form [13]. These data reconfirm that the peptide mimotopes effectively immunologically mimic the neolactoseries structures, inducing a humoral immune response cross-reactive with neolactoseries expressing human tumor cell lines.

Tumor cell cytotoxicity

Antibody mediated cell killing is one of the immunological mechanisms for tumor cell destruction. Anti-carbohydrate antibodies might mediate complement-dependent-cytotoxicity (CDC) better than cytotoxicity associated with various effector cells [14]. To assess the functionality of the anti-peptide serum against tumor cells, we examined CDC mediation of serum in the presence of human complement, targeting the LeY positive MCF7 human breast adenocarcinoma cell line (Figure 1). Serum raised to a pentasaccharide LeY-conjugate mediates the killing of this cell line in vitro out to 1:80 titer [13]. Similarly, detectable lysis was observed titering up to 1:80 serum dilution for serum raised against the peptide mimotopes, and against synthetic LeY-PAA (Figure 1). The anti-K61104 sera was as efficient as the anti-LeY-PAA sera in mediating CDC, while the anti-K61105, anti-K61106 and K61107 sera displayed a higher capacity for cytotoxicity than the LeY-PAA form. Immunization of mice with MCF7 cells also results in a predominate IgM response that leads to significant CDC mediation of the MCF-7 tumor line. No lysis was observed with pre-immune sera (<1:10), or with the anti-ganglioside antibody ME361. MCF7 is reactive with the anti-LeY monoclonal antibody BR55-2 to which BR55-2 was raised [15-17]. These data indicate that serum generated to carbohydrate mimicking peptides have the potential to recognize LeY and display a required functionality.

Induction of memory response

In studies relevant to understanding mechanisms associated with the utility of peptide mimotopes for vaccine efficacy, priming with mimicking peptides might establish beneficial memory responses for the induction of long-lasting carbohydrate cross-reactive IgM responses. It is observed that mice primed with the human breast tumor cell line MCF7 do not mount a substantial immune response after the primary immunization (Figure 2). A subsequent boost results in an enhanced anti-LeY immune response (Figure 2). To test if the peptides prime for an anti-LeY response, mice were immunized 3 times with a low dose (25ug) of the respective peptides with QS-21 as adjuvant, followed by boosting 1 time with MCF7 cells (without adjuvant) two weeks after the last peptide immunization. Immunization with peptides at this dose result in an anti-LeY response similar to that observed with primary immunization with MCF7 cells. Immunization with higher doses results in serum with higher LeY reactivity in ELISA assays [10-12]. Upon subsequent boost with MCF7 cells, a two-fold increase in LeY reactivity is observed (Figure 2) for peptides 104,105 and 107.

Priming with the WRY containing peptide 106 parallels the four-fold increase in the anti-LeY response observed with boosting of MCF7 primed mice with MCF7. The isotype was again predominately IgM in all cases. Response to LeY is also observed in mice primed with peptide (104) that have rested for 6 months and then boosted 1 time with the MCF7 line. LeY reactivity was the same as that observed for 105 and 107. The reciprocal experiment in which mice primed with MCF7 cells are boosted with peptide did not lead to enhanced anti-LeY serum reactivity (data not shown). Consequently, this data is suggestive that peptide mimotopes can prime for memory responses directed toward tumor cells expressing LeY.

Tumor Challenge

Further evidence for in vivo functionality of peptide mimetic vaccination comes from tumor challenged mice. LeY is not expressed in mice. Subsequently, a mouse model is not yet available to study the in vivo functionality of mice primed with peptides and then challenged with LeY expressing tumor. However, Balb/c mice do express sLeX. An anti-Id made against the monoclonal antibody FH6 has proved to be an effective mimic for sLeX, increasing the median mouse survival time of anti-Id immunized Balb/c mice after tumor challenge with the fibrosarcoma Meth-A tumor cell line [18]. A peptide with the sequence ISDGTTYTYYPDS derived from CDR2 of the heavy chain of the anti-Id appears to be responsible for a portion of this anti-tumor response [18]. This anti-Id derived peptide displays homology with our aromatic peptides in being composed of Tyr residues and displaying homologous hydroxyl groups on the Thr residues. Consequently, we asked if our peptide mimotopes would produce a significant anti-tumor effect in Balb/c mice when challenged with Meth-A tumor cells.

We first examined the survival of groups of host mice immunized with peptide mimotopes compared with control immunizations, followed by challenge with sLeX expressing Meth-A cells (Table 3). Ten days after the third immunization, mice were challenged subcutaneously (sc.) with 10^6 live Meth-A cells (day 0). Survival times of host mice were monitored. The results in Table 3 indicate that pre-immunization with mimicking peptide induced an immune response that prolonged survival time. The p value was <0.001 for the K61106 peptide when the generalized Wilcoxon test was performed on the differences between the survival of the group pre-immunized with peptide and that of the group pre-immunized with a control peptide, or that of the group receiving no treatment.

The above studies were designed as a survival study using a high concentration of tumor cells (10^6). We subsequently initiated a kinetic study using a low, $5X10^4$, and moderate $3X10^5$ concentration of Meth A tumor cells. Mice were again immunized 3 times with mimicking peptides, followed by sc challenge with Meth A cells (Figure 3). The growth kinetics indicate that tumors grew slowly or not at all when peptide immunized mice were challenged with tumors within this dose range, suggesting that protection is dependent upon the concentration of tumor cells given to the animal. These data are further suggestive that peptide mimotopes of carbohydrates can elicit beneficial anti-tumor immune responses.

Discussion

The pattern of carbohydrate antigens expressed by different tumor types has been established, paving the way for polyvalent-antibody inducing vaccines [1,19]. The basis for carbohydrate based cancer vaccines that primarily induce an antibody response is well established in both experimental models and the clinical setting [1,6,13,19-30]. It is the expectation, based upon evidence from carbohydrate vaccination trials [24,25,31-36], that antibodies can play a role in vivo in tumor regression, potentially opsonizing tumor cells to prevent extravasion, intravasion and metastatic potential. It is envisioned that antibody induction is especially effective in the adjuvant setting when the targets are circulating tumor cells and micrometastases [37,38]. In general terms, with regard to a major objective of carbohydrate vaccines--i.e. generation of cytotoxic antibodies--variability in patient response and lack of persistence of high-titered IgM cytotoxic antibodies in many patients immunized with carbohydrate-conjugates are problems that remain to be solved and emphasizes the still current limitation of this type of approach.

Molecular mimicry of carbohydrate antigens by peptide mimotopes is one way to augment immune responses to carbohydrate antigens in that they are intrinsically T cell-dependent antigens. We have shown that peptide mimics of neolactoseries antigens can induce IgM antibodies that cross-react with natively expressed LeY and sLeX antigens on the human tumor surface (Table 2). It is often thought that IgM may not be therapeutically beneficial in spite of consistent documentation of the clinical benefit of anti-ganglioside IgM antibodies [6]. This misconception is based on the immune response to protein antigens, which suggest that the IgM response is only transient and not persistent. The basis for attempting to create vaccines capable of inducing higher titer and long-lasting IgM responses against human tumor associated carbohydrate

antigens is the clear correlation of elevated IgM production with improved patient survival [36]. IgM changes from "planar" to "staple" conformation when it binds to clustered epitopes. The "staple" conformation facilitates complement fixation and complement-mediated lysis. Serum containing predominately anti-LeY IgM is functional as measured by CDC in the presence of human complement (Figure 1), overcoming the known inefficiency of homologous complement lysis. An important feature of any vaccine is to induce long term memory responses. We observed that priming mice with mimicking peptides establishes memory responses, up to six months, that may be beneficial for the induction of long-lasting carbohydrate cross-reactivity (Figure 2).

The Meth A sarcoma is one of the best studied of all murine tumors. It is found that a CD4+ T cell-mediated delayed-type hypersensitivity (DTH) response activating non-specific killer cells such as macrophages, NK and LAK cells, without a specific CD8+ cytotoxic T lymphocyte (CTL) response, is the major immune response leading to Meth A tumor rejection in primary immune mice [39]. Humoral responses against Meth A cells is also attributed as a basic immunological mechanism underlying tumor rejection in challenge studies when an anti-idiotypic antibody that mimicked sLeX was used as the primary immunogen [18]. In those studies antibody-dependent cytotoxicity (ADCC) was suggested as the mediator of tumor killing. Vaccination with the peptide mimotopes indicates a humoral response directed toward sLeX which is expressed on Meth A cells (Table 2). As in the anti-Id studies, peptide mimotope immunized mice display increased survival time after tumor challenge with the Meth-A-sarcoma line (Table 3). To better clarify the meaning of the immunization, the kinetics of tumor growth was measured in each immunized mouse by measurement of tumor diameters twice a week. Not unexpectedly graded challenges with lower tumor doses indicate that tumor rejection is dependent upon the concentration of tumor cells in the challenge. Immunization of mice with peptides followed by boosting with Meth A cells did not lead to enhanced antibody titers reactive with sLeX or Meth A cells as assessed by ELISA and FACs analysis respectively (data not shown). Further mechanistic studies are necessary to determine the extent to which the mimotopes mediate T cell responses to Meth A and the role played by IgG mediated ADCC.

It is possible that our peptide mimotopes are also mimicking an as yet unknown tumor associated protein antigen on Meth A. Recently, a peptide that mimics the Gal epitope was shown to induce MUC1

cross-reactive cytotoxic T cells expressed on MCF7 cells, recognizing a peptide region on MUC1 that shared sequence homology with the Gal mimic [3]. The issue is not whether peptide mimotopes of tumor associated carbohydrates can be defined, but under what conditions will peptide mimotopes are beneficial for clinical utility. In addition to the role that peptide mimotopes can play in exploring the fine specificity of antibodies, they may mimic polysaccharides as immunogen and potentially elicit an anti-polysaccharide response. Not all peptides identified as antigen mimics induce polysaccharide cross-reactive immune responses [40]. The problem now is to determine ways to modify such peptides to induce a high anti-polysaccharide response and then harness their intrinsic ability to mediate T cell responses for tumor cell killing. Peptide mimotopes for carbohydrate antigens open a new arsenal for targeting a set of long-standing tumor associated antigens that are understudied.

Experimental
Materials and Methods

Preparation of peptide antigens.

Peptides (Table 2) were synthesized as Multiple Antigen Peptides (MAPs) (Research Genetics, Huntsville Alabama) made by Fmoc synthesis on polylysine groups resulting in the presentation of 8 peptide clusters.

Preparation of Antibodies against Carbohydrate-Mimicking Peptides and LeY

For generation of polyclonal sera, Balb/c mice (n=4 per group) 4-6 weeks of age, were immunized i.p. with 25-50 ug of the respective MAPs and 20ug of QS-21 adjuvant (Aquila Pharmaceuticals, Worcester MA), at intervals of 2 weeks for 6 weeks. Likewise, LeY incorporated into polyacrylamide (PAA) matrix, (creating 30kDa multivalent polymers (GlycoTech Inc.)) was used as an immunogen with QS-21. Balb/c mice were also immunized with the LeY human breast tumor cell line MCF7, without QS-21. Serum from all immunized mice was collected at 7 and 14 days after the last immunization and stored at -20^{o}C.

Flow cytometry

Representative human LeY expressing cell lines includes the breast cancer lines SKBR3, SKBR5, MCF7, and OVAR-3 obtained from ATCC (Rockville, MD). Control cell lines include the human non-LeY expressing cell line HS578 Bst (normal breast cell line, ATCC), and the human melanoma line WM793 (gift from D. Herlyn, Wistar Institute), SKMEL- 28 (ATCC) and NIH3T3 murine fibroblast. For the preparation of cells, 10ml of FACS buffer was added and the cells were washed, scrapped and transferred to 15 ml.

centrifuge tubes. Viable cells were counted using trypan blue as indicator. Cells were diluted to 2 $X10^6$/ml and 100ul used for each sample. Primary sera (10ul) was added to the sample tubes and incubated on ice for 30 min. washed twice with 1 ml FACS buffer and centrifuged for 5 minutes at 1500 rpm. 10 ul of FITC Ab (goat anti-mouse IgG or IgM FITC labeled (Sigma) diluted 1:20 with PBS) was added to the sample and incubated on ice for 30 min. and again washed twice with FACS buffer. Cells were fixed using 2% paraformaldelhyde, followed by FACS measurement on a Becton Dickinson flow cytometer FACScan (Becton Dickinson, Los Angeles CA).

Complement-dependent cell cytotoxicity (CDC): Briefly, a serum was tested for its ability to bind to tumor lines and modulate CDC as previously described [12,41]. Ten ul of each Cell line ($4X10^4$ cells per ml) were added to triplicate wells of a microtiter plate to which was added serially diluted sera, and incubated on ice. Human complement (Sigma) 1:4 was added and allowed to incubate for 4 hrs at 37°C. Tests were done in duplicate with medium, sera, and complement controls. The medium was discarded and 50 ul methanol fix was added and allowed to incubate for 10 min. This procedure was repeated, once again for 5 min. The number of viable cells was determined by Giemsa staining. Plates were counted under a light microscope. The percent of cytotoxicity (PC) is calculated with the formula: Percentage cytoxicity=[1-(number of cells in well treated with antibody and complement/number of cells in well treated with medium only)] x 100. Control wells did not contain anti-sera.

Ig isotype composition and antigen reactivity of anti-peptide sera: Sera isolated from blood samples of pre-immunized and immunized mice were assayed by quantitative ELISA using microtiter plates coated with LeY-PAA or sLeX-PAA (2ug/well) (GlycoTech Inc.)) [12]. The assay was developed using non-specific secondary antibodies to measure total responses and isotype-specific secondary antibodies to monitor the contribution of IgM, IgG1, IgG2a, and IgG2b and IgG3 components. Reciprocal end-points of each serum were used as an indicator of antibody responsiveness.

Tumor challenge

In these experiments, groups of Balb/c mice were immunized i.p. either with 25ug or 100ug of the respective peptides with QS-21 (Table 1), three times at 2-wk intervals. We examined the survival of groups of host mice pre-immunized with the respective MAP peptides, with QS-21 as adjuvant, compared with control

immunizations, followed by challenge with sLeX expressing Meth A cells. Separate groups were immunized with control carrier protein or adjuvant. Ten days after the third immunization, mice were challenged subcutaniously (sc) with 10^6 live Meth-A cells expressing sLeX antigen (day 0). Survival times of host mice were monitored and statistically assessed using the generalized Wilcoxon test [18]. For the kinetic growth studies, groups of mice (n=4) mice were vaccinated with 100 ug of mimotope and QS-21 and challenged sc with $3.1X10^4$ or $5x10^3$ Meth A cells. Tumor diameter was measured twice a week.

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Table 1. Peptides used in these studies

Peptide Name	Sequence	Source
1105	GGIYYPYDIYYPYDIYYPYD	Designed Synthetic
1106	GGIYWRYDIYWRYDIYWRYD	Designed Synthetic
1107	GGIYYRYDIYYRYDIYYRYD	Designed Synthetic
1104	IMILLIFSLLWFGGA	Isolated from peptide library with BR55-2
C1	GDTRYIPALQHGDKK	Irrelevant Control

Table 2. Reactivity of Various Anti-Peptide Sera and Monoclonal Antibodies with Neolactoseries Expressing Cell Lines As Measured by FACS

Cell Lines	Anti-1104	Anti-1105	Anti-1106	Anti-1107	FH6	ME361	BR55-
SKBR3	443/138*	326/128*	418/133*	445/140*	ND	9 (0.1ug)	144 (0.1
MCF7	560	593	595	538	42(0.5ug)	ND	352
PC-3	118	141	129	330	420 (0.1ug)	14 (.1ug)	28(0.1u
Meth-A	183	87	124	143	430 (0.1ug)	15 (0.1ug)	15 (0.1u
SKMEL-28	ND	47.0	33.0	49.8	ND	26 (0.1ug)	7 (0.1ug

Final Sera Concentration is 1:50. Background Mean Fluorescence associated with non-specific mouse sera is on average 24.2. The asterisks indicate IgG portion of anti-peptide serum reactive with SKBR3 cell line. ND is not determined.

Table 3. Survival of groups of host mice pre-immunized with peptide compared with control immunizations followed by challenge with sLeX expressing Meth A cells.

Immunogen	Nominal antigen mimicked	Number of mice immunized	Survival Time (months)	Statistical significance compared to:	
				None	C1
1105	Le	12	2.44±1.23	p<0.05.	p<0.05
1106	Le	12	3.60±1.56	p<0.001	p<0.005
1107	Le	12	2.40±1.32	p<0.05	p<0.05
G1	GD2/GD3	11	1.45±0.33	N.S.	N.S.
QS-21	-	11	1.51±0.43	N.S	N.S.
Proteosome	-	11	1.48±0.42	N.S.	N.S.
C1	-	11	1.44±0.22	N.S.	-
None	-	12	1.46±0.26	-	N.S.

None is tumor alone. N.S. Not Statisticaly significant.. Proteosome is immunologic carrier control derived from meningococcal outer membrane proteins. The C1 peptide was coupled to the proteosome carrier formulation. G1is a control MAP peptide that mimics another tumor associated carbohydrate antigen.

Figure Legends

Figure 1. Titration of Complement-Dependent Cytotoxicity of MCF7 cells. Sequences of peptides 104, 105, 106 and 107 are shown in Table 1.

Figure 2. Priming responses to peptide mimotopes. Peptides used in this study correspond to those shown in Table 1 synthesized as MAP peptides. No adjuvant was used with MCF7 cells. Serial dilution corresponding to 1:50 (shown in A), 1:200 (B) and 1:800 (C) are shown for ELISA reactivity with sLeX synthetic probe.

Figure 3. Tumor growth of peptide immunized mice. Mice were pre-immunized with peptide and then challenged with sLeX expressing Meth A tumor cells.

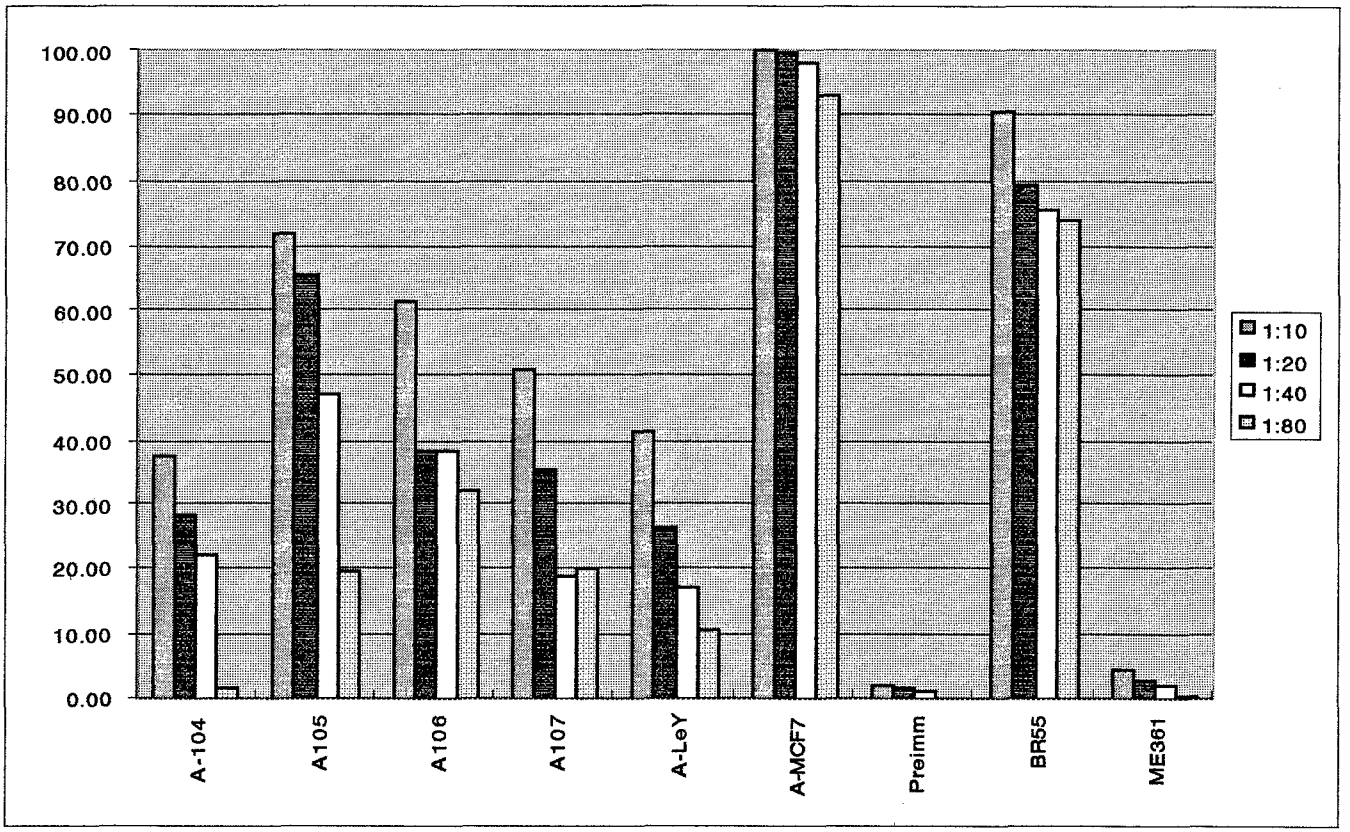
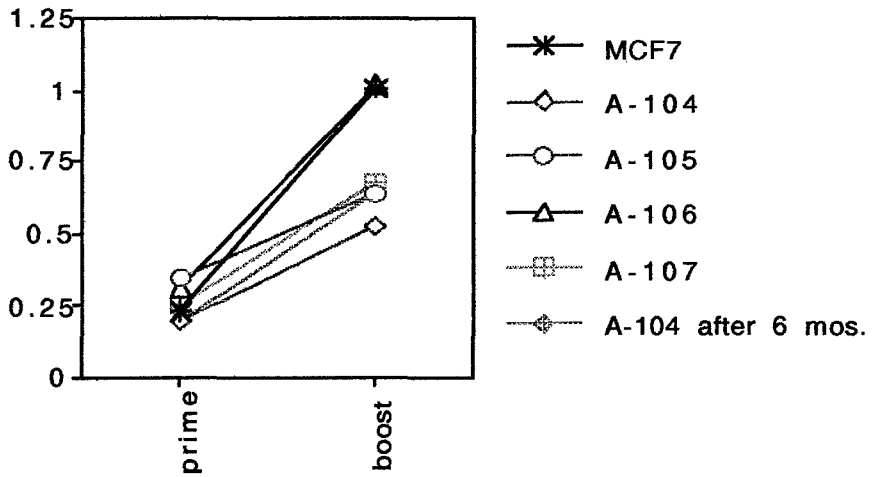
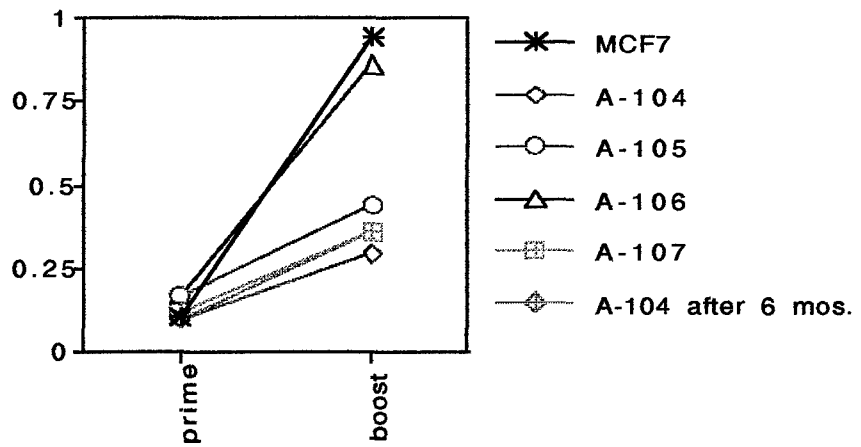

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
A-104
A105
A106
A107
A-LeY
A-MCF7
Preimm
BR55
ME361
1:10
1:20
1:40
1:80

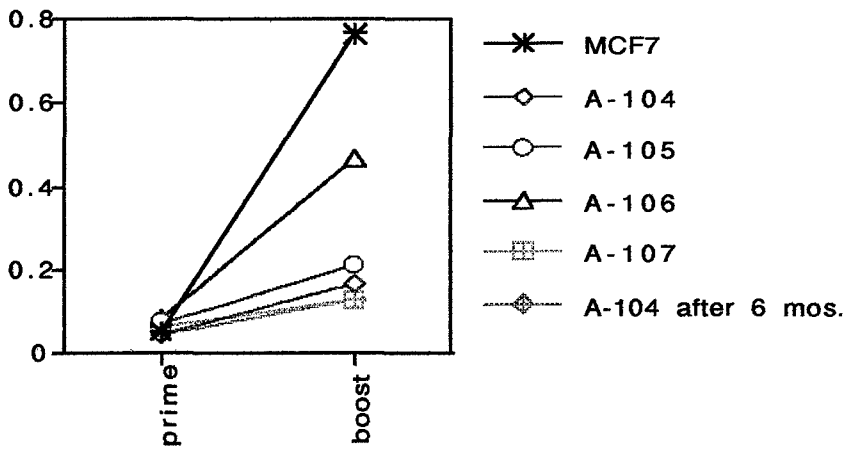
Figure 1.


1.25
1
0.75
0.5
0.25
0
prime
boost
MCF7
A-104
A-105
A-106
A-107
A-104 after 6 mos.

A.


1
0.75
0.5
0.25
0
prime
boost
MCF7
A-104
A-105
A-106
A-107
A-104 after 6 mos.

B.


0.8
0.6
0.4
0.2
0
prime
boost
MCF7
A-104
A-105
A-106
A-107
A-104 after 6 mos.

C
Figure 2

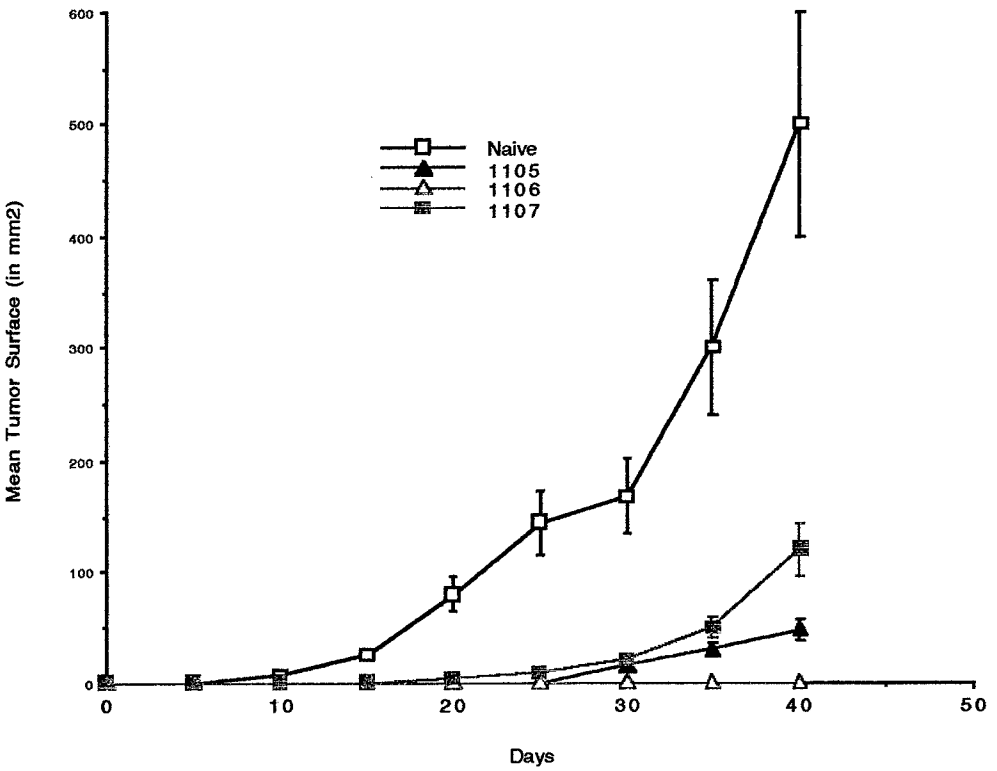

Mean Tumor Surface (in mm2)
600
500
400
300
200
100
0
Naive
1105
1106
1107
0
10
20
30
40
50
Days

Figure 3.

Rec'd
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